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Induratio penis as a Complication to Penis Tuberculide

By

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The first report on penis tuberculide was reported by Mori in Japan in 1891, and Yanagihara[1] gave out a detailed report on 32 cases of it in 1919 and proposed to accord a distinct place to the tuberculide of penis or glans penis among the cutaneous tuberculides. Since then, more than 200 cases have been reported in Japan, so that it is scarcely looked upon as of rare occurrence in Japan, but in Europe it is chiefly described as papulonecrotic tuberculide of glans penis and is deemed as a rare malady.

Of the total 558 patients of cutaneous tuberculosis treated at this Clinic in 43 years of 1910 to 1952, 48.75% or 272 cases suffered from tuberculides as shown in Table I. In contrast to the prevalence of lupus vulgaris in Europe, it is seen that tuberculides are so frequent in Japan.

TABLE I

Total Cases of Patients with Cutaneous Tuberculosis in our Clinic (1910–1952)

Diagnosis	Cases	
Lupus vulgaris	141	
Scrofuloderma	54	
Tuberculosis verrucosa cutis	50	46.24%
B.C.G. Ulcer	9	
Tuberculosis miliaris ulcerosa	4	
Tuberculides		
Lupus miliaris disseminatus faciei	56	
Papulonecrotic tuberculide	41	
Penis tuberculide	18	48.75%
Lichen scrofulosorum	21	
Other tuberculides	21	
Groupe of Lupoid	28	5.01%
Total cases	558	

18 of the tuberculide cases were of the penis type, and it seems natural that Toyama[2] and Yanagihara have called this disease indigenous to Japan and that there should be a Japanese name corresponding to penis tuberculide. Sometimes, it has been reported, induratio penis accompanies this disease, and 16 cases of such a complication have been reported in Japan. Among the 18 cases of penis tuberculide treated at this Clinic, 5 were found to have the induration. But histological study on such an induration has been undertaken in only very rare cases. Recently, I had the opportunity of observing 2 cases of such indurations and in one case amputatio penis was carried out by wish of the patient, so that the induration could be made a minute histological examination, of which I will report the results in the following.

Case Report

Case 1: Male, 31 Years; first examination: October 7, 1952; family history: Wife and one child healthy, nothing special to mention; anamnesis: Had peritonitis at the age of 7, diagnosed as having ulcus molle at 19, received appendectomy at 21 years old. Present history: At the age of 26 millet size papules appeared on his glans penis, which gradually ulcerated. Diagnosed as penis tuberculide at this Clinic and cured by radium therapy. Since then, had two recurrences. In October, 1952, ulcers developed in glans penis, causing pain at urination, for which the patient came to this Clinic for consultation. At present, general conditions good; heart and lungs healthy; Mantoux's reaction positive; blood sedimentation rate: 5–12 mm; X-ray of chest normal. Findings on urogenital organs: some minor scars on the back side of the glans penis, 3 small ulcers of half grain size to whole grain size around the urethral orifice, with the surface partly covered with greyish yellow crusts and feeling light pain upon pressure; no outward anomaly in penis trunk, but an induration was felt by touch in the interior, running from the root of the penis up to the corona of the glans, a little to the right of the median line, of the hardness of cartilage. No pain was caused by the induration, even upon pressure nor by erection, but the penis was seen to bend slightly to the right when erect. Both the inguinal glands were swollen to a size of peas, but nothing abnormal was observed in the testicle, the epididymis and the prostate. Wassermann's reaction, Murata's reaction and Kahn's reaction all negative, Frei's reaction also negative, urine and feces normal, liver function test by Takata pseudo-positive. Blood test: erythrocytes, 4.23 mill.; leucocytes, 6,800; hemoglobin, 98% (Sahli's method); neutrophil cells, 34%; lymphocytes, 55%; monocytes, 4%; eosinophil cells, 2%, showing some trace of relative lymphocytosis. Applying isonicotinic acid hydrazide in total

dosis of about 15 g. in 60 days, the penis tuberculide was cured leaving scars, but the induration in the penis was not effected by the treatment.

Case 2. Male, 49 years; first examination, November 19, 1952; family history: wife and three children healthy; anamnesis: Beriberi at the age of 16, paratyphoid fever at 19 and diagnosed of pulmonary infiltration at 42 years old. Present history: Induration began at the penis trunk when patient was about 34 years old and gradually grew to the vicinity of the glans penis but no pain was caused. Four years before, in spring, pustules of millet size to grain size formed on the glans penis causing pain and was treated at a certain hospital as penis tuberculide and induratio penis plastica with radium and cured almost completely. In the spring of 1952, eruption reappeared on the glans penis, which gradually ulcerated and began to cause pain at urination. Upon first examination at our Clinic, the patient showed good general conditions but some paleness in complexion, indicating anemia. Heart and lungs healthy; Mantoux's reaction positive; blood sedimentation rate: 4–7 mm; X-ray of chest showed a calcified focus in the right lobus; blood pressure: 120–80 mm. Hg; patellar reflex: normal; findings on urogenital organs: many scars as if of worm bites were seen on the dorsal side of the glans penis; around the urethral orifice, 2 ulcers, 11×4 mm and 9 mm. $\times$ 6 mm. in size, were found covered by greyish yellow crusts, extremely sensitive to stimuli and causing marked pain upon pressure. Urination caused particularly severe pain at the sites of the ulcers. The trunk of the penis showed nothing abnormal in appearance, but in the interior, in particular, in the right corpora cavernosa penis, an induration was felt, which extended from the corona glandis to the root of the penis, cartilageous in hardness but causing no pain nor curving by erection. Both the inguinal glands were swollen to the size of small finger tip, but the testicle, the epididymis and the prostate were unaffected. Wassermann's reaction, Murata's reaction, Kahn's reaction and Frei's reaction all negative, urine and feces normal, liver function test by Takata negative. Blood test showed erythrocyte count, 3.09 mill.; leucocytes, 5,800; hemoglobin, 80% (Sahli's method) neutrophil cells, 60%; lymphocytes, 35%; monocytes, 3%; eosinophil cells, 2%; indicating slight anemia. Treated with isonicotinic acid hydrazide in the total dosis of 12 g. in about 50 days, the penis tuberculide was nearly cured but the induration was not bettered. By the wish of the patient, the penis was amputated, so that the parts affected by the tuberculide and the induration could be studied histologically.

Histological Observations: The series of penis sections were stained with hematoxilin-eosin, by van Gieseon's method, by Weigert's method and by Ôno's method belonging to the mucopolysaccharide staining

methods, for microscopic examination. The tuberculide had been clinically nearing cure and histologically no marked tubercles were found. The main findings were some infiltrations of round cells, sparsely surrounded by plasma cells and histiocytes, mingled with a few epitheloid cells, showing rather the appearance of productive change. Examination of a cross-section of the penis trunk showed that tubercles first formed at the septum, gradually undergoing caseous degeneration, then spreading to the corpora cavernosa penis, the right corpus being most affected, and also to the corpora cavernosa urethrae. Everywhere, conspicuous tubercles composed of epitheloid cells and Langhans's giant cells are found, especially well developed around the central blood vessels. Around the arteria profunda penis, thrombophlebitis and canalization of veins was observable, indicating the probable presence of thrombophlebitis tuberculosa, but our search for tubercle bacillus in tissues gave only negative results. My assumption is that in this case tubercle bacillus and their products had been hematogenously disseminated to give rise to penis tuberculide with induratio penis accompanying it.

Discussion

In 1919, Yanagihara[1] classified penis tuberculides or tuberculosis of penis in its broader sense, as follows: 1) cases showing eruptions on the glans penis, in the size of a millet to that of a pin-head (folliculitis), 2) cases with no surface symptoms but with induration in the interior of the penis, to the size of a pea or even larger in some case (acnitis), 3) cases with the induration affecting the glans penis and the corpora cavernosa penis and urethrae as well, 4) cases with induration in the subcutaneous veins of the penis, 5) cases affecting the skin of the penis trunk, thus covering all cases of indurations caused by tuberculosis under one heading of penis tuberculides. Hellerström[3] and Granroth[4] had reported two cases each of penis tuberculide. Toyama had also described induratio penis as a complication to penis tuberculide. On the other hand, there have been reported many cases of induratio penis plastica in the field of dermatology and urogenital pathology, since La Peyronie first reported it in 1743, but there have been no agreement on its cause. Napolic and Ferdinand proposed their tuberculosis theory. In Japan, K. Dohi also deemed it as in most cases accompanying tuberculide and consequently as tuberculous in genesis, though in a fewer cases the induration may occur independently. Komuro[5] states that induratio penis as complication to penis tuberculide should not be diagnosed as induratio penis plastica, but Ôgushi[6] contends that there is no denying that some cases of induratio penis plastica caused by tuberculosis do exist. From the histopathological point of view, induratio penis plastica in most

cases are free of inflammatory change. Rothschild has observed groups of fusiform cells around the small blood vessels of such tissues which he called " Bildungszellen " and assumed them to be the source for forming indurations capable of direct development into connective tissue, cartilaginous tissue and osseous tissue. In Japan, there were more of cases with osseous tissue formation, calcium deposition and specific cells, only five cases including the above described two coming under my observation being subject to tuberculous genesis. However, there is as yet nothing certain on the cause of such indurations, as mentioned above, and though there is little ground for excluding tuberculous indurations from indurations from induralio penis plastica, histologically speaking, at least, it is more convenient clinically to place indurations accompanying tuberculous changes under a separate heading of induratio penis tuberculosa. For treatment, isonicotinic acid hydrazide and such chemicals were used with advantage against penis tuberculide but almost to no purpose against the accompanying induratio penis, which should be treated with radium or X-ray, in the same manner as induratio penis plastica.

Conclusion

1. Two cases of induratio penis occurring as a complication to penis tuberculide were observed and in one case, histological study could show the existence of unmistakable tuberculous change at the indurated portion, which could be presumed to be hematogenous in nature.

2. It is clinically more convenient to treat induratio penis accompanying tuberculous genesis under a separate heading from induratio penis plastica, as induratio penis tuberculosa.

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Studies on Blood Glycogen

The 4th Report

Blood Glycogen Content in Diabetes mellitus and Alloxandiabetes

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Since Gabritschewsky[1], many investigators agree in their opinion that the blood glycogen content of diabetes mellitus patients show in general higher values than healthy persons. As regards the blood glycogen content of the pancreatectomized dog Kaufmann[2] recognized its increase, but Fahr[3] denied such elevation. According to Prof. Okamoto[4], the permanent alloxandiabetes closely resembles the diabetes mellitus.

Therefore, we attempted in the present experiments to estimate the blood glycogen content of diabetes mellitus patients and alloxandiabetic rabbits, in order to clarify its clinical significance.

Experimental

Method

The blood glycogen content was estimated by our own micromethod[5] and the blood sugar content by Fujita and Iwatake's method.[6] In order to produce alloxandiabetes in rabbits, we injected intravenously 5% alloxan ("Daiichi") 200 mg. per kg. According to our former experiments[7], the normal range of the human blood glycogen content is from 30 to 74 mg. % and that of rabbits from 20 to 80 mg. %.

Results

1. Relation of the blood glycogen content to the form of diabetes mellitus

According to the classification of our Clinic[8], diabetes mellitus patients can be divided into two forms: Type I can be cured only by our diet therapy (carbohydrate 300–350 g., fatt 20–50 g., protein 70–100 g.) and Type II can be cured by the same diet therapy with insulin injection.

As shown in Table I and II, 20 cases belong to Type I and 25 cases

TABLE I

Blood Glycogen Content of Type I of Diabetes mellitus

No.	Name	Sex	Age	Blood glycogen (mg%)	Blood sugar (mg%)	Complication
1	T.K.	♂	53	91	146	–
2	Y.N.	♀	48	87	151	–
3	Y.K.	♂	47	85	174	–
4	M.O.	♂	43	81	176	–
5	Y.H.	♂	50	78	174	–
6	G.O.	♂	47	70	92	–
7	H.S.	♂	38	68	198	–
8	T.W.	♀	45	66	153	–
9	H.S.	♂	50	65	181	–
10	T.O.	♀	60	61	117	–
11	M.F.	♂	40	61	91	–
12	T.W.	♂	42	60	190	–
13	S.S.	♂	55	59	200	+
14	S.O.	♂	50	54	146	–
15	S.S.	♂	55	54	141	–
16	S.I.	♂	51	49	141	–
17	K.K.	♂	47	48	141	–
18	M.C.	♀	52	47	125	–
19	T.I.	♂	59	38	110	–
20	S.I.	♂	65	36	122	–

to Type II. The results of the examination are given in Table III: 5 cases of Type I revealed high values of over 75 mg. % (25%), but 8 cases of Type II showed high values (32.1%) and 2 cases of them lower values than the normal value (7.9%). Consequently, 13 cases of the whole patients presented higher values (28.9%) and there was no evident difference between Type I and II, but the blood glycogen content of Type II had a tendency not only to decrease, contrary to Type I, but also to increase when its complications occurred.

2. Relation of the blood glycogen content to the process of diabetes mellitus

As presented in Table IV, the blood glycogen content of Type II of diabetes mellitus has a tendency to become normal at the same time that the blood sugar normalizes, with the exception of the normal value in the initial stage. In these cases insulin works in general to decrease the content of blood glycogen in company with the diminution of the blood sugar.

Therefore, the blood glycogen content seems to become normal as

TABLE II

Blood Glycogen Content of Type II of Diabetes mellitus

No.	Name	Sex	Age	Blood glycogen (mg%)	Blood sugar (mg%)	Complication
1	H.I.	♂	52	114	252	−
2	I.K.	♂	50	105	264	−
3	K.M.	♂	43	93	178	+
4	H.S.	♂	53	88	274	−
5	Y.Y.	♂	50	87	346	+
6	T.Y.	♀	48	79	142	+
7	T.W.	♀	59	78	215	+
8	B.K.	♂	58	78	150	−
9	S.S.	♂	34	74	216	−
10	T.S.	♂	66	73	282	+
11	N.M.	♀	18	69	272	−
12	K.A.	♀	14	69	214	−
13	N.K.	♀	53	66	294	−
14	S.W.	♂	44	65	145	−
15	E.A.	♀	58	63	310	−
16	R.T.	♀	35	62	349	−
17	S.K.	♀	46	57	207	−
18	H.K.	♀	50	52	254	−
19	C.S.	♀	34	51	314	−
20	M.Y.	♂	49	48	112	−
21	K.K.	♂	53	44	177	−
22	M.W.	♂	45	43	298	+
23	T.S.	♀	47	30	147	−
24	K.M.	♂	23	24	178	−
25	M.F.	♂	58	23	186	−

the condition of patients shows good turns for recovery by means of insulin injection.

3. Behaviour of the blood glycogen content of alloxandiabetes

According to a secondary hyperglycemia produced by alloxan injection, we classify the alloxandiabetes into 3 forms: a complete form, in which the hyperglycemia over 200 mg. % continues more than 4 weeks, an acute form, which dies within a week under intensive hyperglycemia, in spite of insulin injection, and an incomplete form, in which the blood sugar becomes normal within a week.

The results presented in Table V show that the blood glycogen content is almost unchanged in the first stage, and it tends to increase when alloxandiabetes has occurred. But it decreases occasionally before the

TABLE III

Relation of the Blood Glycogen Content to the From of Diabetes mellitus

Form	Complication	Elevated	Normal range	Decreased	Total
Type I	−	5	14	0	19
	+	0	1	0	1
	Total	5	15	0	20
	%	25.0	75.0	0	100.0
Type II	−	4	13	2	19
	+	4	2	0	6
	Total	8	15	2	25
	%	32.1	60.0	7.9	100.0
Total	−	9	27	2	38
	+	4	3	0	7
	Total	13	30	2	45
	%	28.9	66.8	4.3	100.0

TABLE IV

Relation of the Blood Glycogen Content to the Progress of Diabetes mellitus

No.	Name		Before diet therapy	After insulin injection		Dose of insulin (units)
				min.	1 hour after	
1	H.I.	Blood glycogen (mg%)	114	35		
		Blood sugar (mg%)	252	161		
3	K.M.	Blood glycogen (mg%)	93	46	63	20
		Blood sugar (mg%)	178	112	102	
11	N.M.	Blood glycogen (mg%)	69	72	58	15
		Blood sugar (mg%)	272	114	76	
15	E.A.	Blood glycogen (mg%)	63	38	42	10
		Blood sugar (mg%)	310	205	127	
16	R.T.	Blood glycogen (mg%)	62	66		
		Blood sugar (mg%)	339	276		
19	C.S.	Blood glycogen (mg%)	51	61	47	15
		Blood sugar (mg%)	325	141	67	
22	M.W.	Blood glycogen (mg%)	43	23		
		Blood sugar (mg%)	298	204		
23	T.S.	Blood glycogen (mg%)	30	32		
		Blood sugar (mg%)	147	118		
24	K.M.	Blood glycogen (mg%)	24	48	26	15
		Blood sugar (mg%)	178	106	91	
25	M.F.	Blood glycogen (mg%)	23	67	57	15
		Blood sugar (mg%)	186	111	54	

death of an acute form.

Thus the blood glycogen content of alloxandiabetic rabbits tends in general to increase, but it decreases occasionally before the death of

TABLE V

Behavior of the Blood Glycogen Content of Alloxandiabetic Rabbits

Form	No.		Before inj.	Time after injection										
				2 hours	4 hours	1 day	2 days	3 days	5 days	1 week	2 weeks	3 weeks	4 weeks	5 weeks
Complete	1	Blood sugar (mg%)	138	309	308	209	326	425	330	351	362	390	372	388
		Blood glycogen (mg%)	56	48	49	64	94	24	44	39	35	38	58	99
	2	Blood sugar (mg%)	107	144	80	252	270	348	320	355	319	323	308	262
		Blood glycogen (mg%)	48	49	47	42	33	33	36	60	56	45	62	57
	3	Blood sugar (mg%)	101	314	270	95	386	500	309	382	282	488	356	384
		Blood glycogen (mg%)	34	39	34	30	20	23	48	41	34	30	41	34
	4	Blood sugar (mg%)	141	211	77	222	383	690	+					
		Blood glycogen (mg%)	56	59	53	46	65	22						
Acute form	5	Blood sugar (mg%)	111	242	220	90	348	452	590	+				
		Blood glycogen (mg%)	38	25	34	48	36	36	16					
	6	Blood sugar (mg%)	122	345	345	418	525	224	448	+				
		Blood glycogen (mg%)	23	26	34	33	16	26	20					
	7	Blood sugar (mg%)	168	342	178	71	225	464	294	135				
		Blood glycogen (mg%)	41	46	26	34	36	43	48	49				
Incomplete form	8	Blood sugar (mg%)	123	201	60	135	251	309	272	177	146			
		Blood glycogen (mg%)	39	48	49	44	34	48	45	79	35			
	9	Blood sugar (mg%)	119	164	99	177	276	258	248	159				
		Blood glycogen (mg%)	39	30	34	30	34	25	36	23				
	10	Blood sugar (mg%)	136	290	159	98	220	348	340	320	116			
		Blood glycogen (mg%)	33	49	43	31	33	81	68	69	62			

an acute form.

DISCUSSION

Many investigators such as Gabritschewsky[1], Schwarz[9][10], Staub[11], Kanda[12] and Okaya[13] reported that the blood glycogen content of diabetes mellitus patients was in general elevated. In these experiments we estimated the blood glycogen content of 45 diabetes mellitus patients and recognized that the blood glycogen content of Type II of the classification of our Clinic has a tendency not only to decrease, contrary to Type I, but also to increase when its complications occur. Moreover, we ascertained that the blood glycogen content of Type II seems to become normal as the condition of patients shows good turns for recovery by means of insulin injection.

According to our former experiments[14], it is obvious that a part of glycogen in the liver pours into the blood as such, when the capacity of the liver to keep glycogen is enfeebled. This is the reason why the

content of blood glycogen is elevated in the cases of diabetes mellitus, because the capacity of the diabetic tissues to keep sugar is enfeebled owing to the diminished secretion of insulin.

As for the blood glycogen content of experimental diabetes, Kaufmann[2] recognized the increased content of blood glycogen in the pancreatectomized dog, but Fahr[3] denied such elevation. But hitherto there has been no report on the blood glycogen content of the alloxandiabetic rabbit. Moreover, there has been no concordant opinion as to the liver glycogen content of the alloxandiabetes, because Gomori[15], Lackey *et al.*[16] asserted its decrease, on the contrary, Miller[17] contended for its increase. In our experiments, it is proved that the liver glycogen content of alloxandiabetic rabbits does not differ from that of normal rabbits, but that the function of the former to make and store the liver glycogen is markedly disturbed.

The results obtained from these experiments show that the elevated content of blood glycogen of alloxandiabetic rabbits agree with that of diabetes mellitus patients, because the capacity of the liver to keep glycogen is enfeebled, on the contrary, the decreased content of blood glycogen of an acute form of alloxandiabetic rabbits suggests the diminished glycogen content of the liver.

Summary

From these experiments on 45 diabetes mellitus patients and alloxandiabetic rabbits it can be concluded as follows:

1. As for the blood glycogen content of Type I of diabetes mellitus according to the classification of our Clinic, 25% of them revealed higher values of over 75 mg. %, but 32.1% of Type II showed higher values than the normal value and 7.9% of the latter lower values. Especially the blood glycogen content of Type II tends increase when its complications occur.

2. As the condition of Type II shows good turns for recovery by means of insulin injection, the blood glycogen content seems to become normal at the same time that the blood sugar normalizes. In these cases insulin works in general to decrease the blood glycogen content in company with the diminution of the blood sugar.

3. In the first stage of the alloxandiabetes the blood glycogen content is almost unchanged and tends to increase when a secondary hyperglycemia occurs, while it decreases occasionally before the death of an acute form.

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Studien über Blutdiastase

VII. Mitteilung

Verhalten der Blutdiastase beim Diabetes mellitus und Alloxandiabetes

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Es ist schon bekannt, dass sich die äussere Pankreassekretion zuweilen beim Diabetes mellitus vermindert, so dass das diastatische Ferment im Blut und Harn des Diabetikers sich manchmal erniedrigt.

Nach unseren früheren Untersuchungen[1] ist es klar geworden, dass der Blutdiastasespiegel eine Rolle des Anzeigers der Pankreastätigkeit spielen kann. Aber Meinungen von Autoren sind nicht übereinstimmend in bezug auf Beziehungen zwischen der äusseren und inneren Sekretion des Pankreas, und sogar zwischen Krankheitsformen und Blut-sowie Harndiastasespiegel von Diabetikern.

Seit Jacobs[2], Dunn *et al.*[3] ist es allgemein anerkannt, dass Alloxan eine Zuckerkrankheit des Kaninchens ganz ähnlich wie beim Menschendiabetes verursachen kann.

Infolgedessen wurde in vorliegender Untersuchung erforscht, wie die Blutdiastase sich sowohl beim Diabetes mellitus als auch beim Aloxandiabetes verhält.

Experimenteller Teil

Methodik

Zur Bestimmung der Diastase im Blut wurde die von uns[4] modifizierte Ottenstein[5]-Baltzersche[6] Mikromethode angewandt, und der Diastasegehalt im Harn wurde nach Wohlgemuth[7] und der Zuckergehalt im Blut nach Fujita-Iwatake[8] bestimmt. Nach der I. und II. Mitteilung[1][9] dieser Arbeit erhielten wir bei Menschen Normalwerte der Blutdiastase zwischen 200 und 400 mg/dl, die der Harndiastase zwischen 16 und 32, die des Blutzuckers zwischen 70 und 110 mg/dl erhalten, ander-

erseits bei Kaninchen Normalwerte der Blutdiastase zwischen 800 und 1400 mg/dl und die des Blutzuckers zwischen 60 und 140 mg/dl.

Ergebnisse

1. Einfluss der Krankheitsformen von Diabetes mellitus auf die Blut-und Harndiastase

In unserer Klinik[10] wurden Diabeteskranke in 2 Formen eingeteilt: die 1. Form ist eine leichte Form, die auch nur durch unsere kohlehydratreiche und fettarme Probekost (Kohlehydrat 300–350 g, Fett 20–50 g, Eiweiss 70–100 g) geheilt, die 2. Form eine mässig schwere Form, die durch obige Probekost mit Insulinbehandlung gebessert wird.

Die Untersuchungsergebnisse von 24 Diabeteskranken sind in Tabelle I zusammengestellt: darunter sind 8 Fälle der 2. Form, 11 Fälle der 1., 3 Fälle von flüchtiger Glykosurie und ein Fall von Nierendiabetes vorhanden.

TABELLE I

Blut -und Harndiastase von Diabetikern

Form	Name	Blutdiastase (mg/dl)	Harndiastase	Blutzucker (mg/dl)	Staubscher Effekt	Komplikation
2. Form	F.A.	776	32	254	–	+
	M.E.	540	32	212	–	+
	K.I.	534	16	124	+	–
	S.S.	373	32	205	+	+
	Y.O.	356	4	358	–	–
	I.N.	322	16	136	–	+
	J.Y.	243	16	215	–	–
	N.W.	116	16	242	–	–
1. Form	K.C.	399	16	191	+	–
	G.S.	398	16	134	–	–
	S.O.	369	8	127	+	–
	K.T.	356		133	+	+
	M.T.	319	32	139	–	+
	H.S.	299		105	+	–
	S.T.	296	16	263	+	–
	H.N.	259	8	118	–	+
	S.S.	248	32	115	+	–
	T.F.	220	8	103	–	–
	M.G.	188	4	218	–	+
Flüchtige Glykosurie	H.I.	467		101	–	+
	M.Y.	376	16	120	+	+
	H.S.	328	16	68	+	+
Nieren-diabetes	I.T.	371		89	+	–

Aus Tabelle II ersieht man, dass die Blutdiastase sich bei der 2. Form um 37.5% vermehrt und um 12.5% vermindert, aber bei der 1. Form nur um 9.1% vermindert, während eine Verminderung der Harn-

TABELLE II

Krankheitsformen von Diabetes mellitus und Blut-sowie Harndiastase

Form	Komplikation	Blutdiastase (mg/dl)				Harndiastase		
		erhöht	normal	vermindert	Total	normal	vermindert	Total
2. Form	–	1	2	1	4	3	1	4
	+	2	2		4	4		4
	Total	3	4	1	8	7	1	8
	%	37.5	50.0	12.5	100.0	87.5	12.5	100.0
1. Form	–		7		7	4	2	6
	+		3	1	4	2	2	4
	Total		10	1	11	6	4	10
	%		90.9	9.1	100.0	60.0	40.0	100.0
Total	–	1	9	1	11	7	3	10
	+	2	5	1	8	6	2	8
	Total	3	14	2	19	13	5	18
	%	15.8	73.7	10.5	100.0	72.2	27.8	100.0

diastase bei der 2. Form 12.5% und bei der 1. Form 40% beträgt.

Also findet man bei Diabeteskranken zuweilen eine Verminderung der Blut-und Harndiastase ohne Rücksicht auf Krankheitsformen, ebenso oft eine Vermehrung der Blutdiastase, besonders bei komplizierten Diabetesfällen.

2. Einfluss der Glukosedoppelbelastungsprobe auf die Blutdiastase

Schon in der II. und III. Mitteilung[1)11)] dieser Arbeit berichteten wir eine Herabsetzung der Blutdiastase durch Hyperglykämie nach Glukosezufuhr. Deshalb haben wir in der vorliegenden Untersuchung erforscht, um das Verhalten der Blutdiastase durch Glukosedoppelbelastungsprobe bei Diabeteskranken zu untersuchen, in ähnlicher Weise wie bei der Staub[12)]-und Traugottschen[13)] Funktionsprüfung des Inselapparats. Wenn der Blutdiastasewert nach der erstmaligen Glukosezufuhr niedriger wird als derjenige nach der zweimaligen, so nennen wir den Unterdruckungseffekt der Blutdiastase (Blutdiastaseeffekt) positiv, den entgegengesetzten Fall negativ.

Die Versuchsergebnisse sind in Tabelle III ersichtlich. Der Blutdiastaseeffekt ist ausnahmslos negativ bei 4 Diabetesfällen ohne Rücksicht auf Krankheitsformen, aber positiv bei 2 Nichtdiabetikern, während ein Staubeffektnegativer Glykosuriker sich als negativ erwies.

Also wird es klar, dass man die Krankheitsformen von Diabetes mellitus nicht nach dem Blutdiastaseeffekt einteilen kann.

3. Verhalten der Blutdiastase von Alloxandiabetes

Um Alloxandiabetes zu verursachen, injizierten wir Alloxan aus "der Pharmazeutischen Gesellschaft Daiichi" 200 mg pro kg in die

TABELLE III

Einfluss der Glukosedoppelbelastungsprobe auf den Blutdiastase -und zuckerwert

Form	Name	Analyse (mg/dl)	Vor. Inj.	Zeit der Blutentnahme nach Belastung (St.)						Effekt
				0.5	1	2	2.5	3	4	
2. Form	J.Y.	Diastase	243	212	229	228	195		203	−
		Zucker	215	311	402	353	373	421	392	−
	S.S.	Diastase	373	352		361	328		331	−
		Zucker	205	307	338	266	289	334	273	+
1. Form	H.S.	Diastase	299	265		270	239		242	−
		Zucker	105	162	176	148	175	165	139	+
	T.T.	Diastase	296	272	245	268	254	264	267	−
		Zucker	263	348	381	345	374	372	308	+
Flüchtige Glykosurie	H.I.	Diastase	467	459		486	416		426	−
		Zucker	100	156	153	77	148	172	73	−
	M.Y.	Diastase	353	270	296	319	312		366	+
		Zucker	90	168	203	149	176	137	111	+
Nichtdia-betiker	R.T.	Diastase	637	554	584	564	563		619	+
		Zucker	90	121	122	106	128	98	104	−
	S.S.	Diastase	570	501		540	521		535	+
		Zucker	90	132	95	83	132	78	75	+

Ohrvene der Kaninchen, die 24 Stunden lang gehungert hatten. In der vorliegenden Untersuchung teilten wir alloxandiabetische Kaninchen in folgende 3 Formen ein: 1) eine vollkommene Form, wobei die Hyperglykämie über 200 mg/dl 4 Wochen lang andauert, 2) eine akute Form, wobei das Versuchstier unter hochgradigem hyperglykämischem Zustand binnen 8 Tagen stirbt, 3) eine unvollkommene Form, wobei sich Blutzucker binnen 8 Tagen unter 200 mg/dl erniedrigt.

Aus den in Tabelle IV niedergelegten Ergebnissen lässt sich Folgendes herauslesen, dass die Blutdiastase des alloxandiabetischen Kaninchens im Stadium der Anfangshyperglykämie und der nachfolgenden Hypoglykämie zwar sehr variabel ist, aber im Stadium der sekundären Hyperglykämie leicht herabsteigt, während die Blutdiastase der akuten Form die Neigung hat, aufzusteigen.

Die Blutdiastase von Alloxandiabetes schwankt also anfangs variabel, aber neigt sich im Stadium der sekundären Hyperglykämie herabzusteigen.

DISKUSSION

Die bisherigen Ergebnisse über die Blut-und Harndiastase von Diabetes mellitus sind sehr verschieden: manche Autoren fanden dabei eine Vermehrung (Leo[14], Bendersky[15], Endriquez[16], v. Strasser[17], Chrometzka

TABELLE IV

Verhalten der Blutdiastase von Alloxandiabetes

Form	Nr.	Analyse (mg/dl)	Vor. Inj.	Zeit der Blutentnahme nach Injektion										
				2 St.	4 St.	6 St.	1 Tage	2 Tage	3 Tage	5 Tage	1 Woche	2 Woche	3 Woche	4 Woche
Vollkommene Form	1	Diastase	1155	- 14	135	-286	-139	- 89	21	-343	-354	-303	- 97	- 45
		Zucker	115	272	120	196	184	324	334	358	344	358	368	410
	2	Diastase	930	506	210	455	311	247	4	175	120	- 28	-106	198
		Zucker	95	74	179	35	289	333	316	320	290	302	276	322
	3	Diastase	649	411	482	321	384	395	177	39	- 84	343	235	151
		Zucker	111	130	49	200	117	276	314	372	270	288	306	270
Akute Form	4	Diastase	1305	- 96	- 21	-171	157	655	-559	†				
		Zucker	155	201	75	56	208	380	684					
	5	Diastase	1169	- 44	166	53	649	719	594	†				
		Zucker	66	195	165	38	62	312	334					
	6	Diastase	888	46	- 42	400	550	692	-450	†				
		Zucker	102	206	194	70	322	178	302					
	7	Diastase	978	92	- 16	- 47	251	368	532	518	376	†		
		Zucker	132	230	68	24	121	224	230	224	246			
Unvollkommene Form	8	Diastase	981	- 5	-197	-200	177	357	364	25	-113	- 21	49	
		Zucker	134	184	176	64	112	232	312	324	222	120	120	
	9	Diastase	1151	-109	-186	-246	160	91	- 19	149	63			
		Zucker	109	208	65	56	89	228	268	320	196			
	10	Diastase	1053	35	-149	- 78	- 87		-321	45	156			
		Zucker	117	242	181	55	200		326	312	101			

und Erlemann[18]), und andere Autoren normale Werte (Schlesinger[19], v. Benczur[20], Corbett[21], Harrison[22], Brill[23], Asayama[24]), jedoch die grosse Mehrzahl von Autoren eine deutliche Abnahme (Lépine[25], Achard[26], Wohlgemuth[27], Moeckel[28], Wynhausen[29], Marino[30], Neumann[31], Euler[32], Schmerel[33], Ottenstein[5], Katsch[34], Brinck[35], Eckardt[36]). Aber Schüler[37] und Brinck[38] bemerkten dabei wechselnde Werte, nämlich bald herabgesetzte und bald erhöhte Werte. Betreffs Beziehungen zwischen Diabetesformen und Blut-sowie Harndiastasewerte fand Asayama[24] keinen Unterschied, und sogar berichteten Harrison und Lawrence[22] keine prognostische Bedeutung für die Bestimmung der Blut-und Harndiastase, während Neumann[31] die prognostische Brauchbarkeit behauptet. Auch in der III. Mitteilung[11] dieser Arbeit bemerkten wir keine Beziehung zwischen dem Staubschen Effekt und den Blut-sowie Harndiastasewerten.

In der vorliegenden Untersuchung fanden wir einerseits bei manchen Diabeteskranken eine Verminderung der Blut-und Harndiastase ohne Rücksicht auf Krankheitsformen als Zeichen der Hypofunktion der

äusseren Pankreassekretion, aber anderseits bei manchen, besonders komplizierten Diabetikern eine Vermehrung der Blutdiastase, im Sinne der Fermententgleisung als Zeichen des Mittreagierens des Pankreas. Diese Ergebnisse entsprechen dem Bericht von Okada[39], dass es keine direkte Beziehung zwischen der Krankheitsschwere des Diabetes mellitus und der Störung der äusseren Pankreassekretion gibt.

Nach peroraler Glukosezufuhr fanden McCaughan *et al*[40] eine Anregung der äusseren Pankreassekretion, aber Okada *et al*[41] beobachteten eine reflektorische Steigerung direkt nach Einbringen von Glukose ins Duodenum, nachher aber eine Hemmung derselben im Anschluss an nachfolgende Hyperglykämie. Schon in der II. und III. Mitteilung[1][11] dieser Arbeit bemerkten wir eine Verminderung der Blutdiastase nach intravenöser und peroraler Glukosezufuhr.

In diesen Verscuhsergebnissen ist der Blutdiastaseeffekt nach Glukosedoppelbelastungsprobe negativ bei Diabeteskranken ohne Rücksicht auf Krankheitsformen, indem der Blutdiastasesturz durch Hyperglykämie bei Diabeteskranken später auftritt als bei Nichtdiabetikern. Mit anderen Worten, der Blutdiastaseeffekt ist positiv bei Nichtdiabetikern, weil Insulin durch Glukosezufuhr reichlich produziert wird.

Nach Prof. Dr. Okamoto[42] wird der Alloxandiabetes des Kaninchens dem Menschendiabetes ähnlich, wenn der erstere anhaltend ist. Betreffs der Blutdiastase der alloxandiabetischen Kaninchen berichtet Maita[43] eine Vermehrung der Blutdiastase.

In diesen Versuchen fanden wir anfangs Schwankungen, dann Verminderung der Blutdiastase im Stadium der sekundären Hyperglykämie als Zeichen der Hypofunktion der äusseren Pankreassekretion.

Also lässt sich vermuten, dass die Blutdiastase beim Diabetes mellitus und Alloxandiabetes sich zur Verminderung neigt, aber bei komplizierten Diabetesfällen sich vermehrt ohne Rücksicht auf Krankheitsformen.

Zusammenfassung

Aus den obenerwähnten Versuchsergebnissen ist das Folgende abzuleiten:

1. Die Blutdiastase vermehrt sich um 37.5% und vermindert sich um 12.5% bei der 2. Form des Diabetes mellitus nach Einteilung unserer Klinik, aber vermindert sich bei der 1. Form nur um 9.1%, während die Verminderung der Harndiastase bei der 2. Form 12.5% und bei der 1. Form 40% beträgt.

2. Der Blutdiastaseeffekt bei Glukosedoppelbelastungsprobe ist negativ bei Diabetesfällen ohne Rücksicht auf Krankheitsformen, aber positiv bei Nichtdiabetikern, ausgenommen einen staubnegativen Glykosuriker, der ein negatives Resultat zeigte.

3. Die Blutdiastase des alloxandiabetischen Kaninchens ist sehr variabel im Stadium der Anfangshyperglykämie und der nachfolgenden Hypoglykämie, aber neigt sich im Stadium der sekundären Hyperglykämie zur Herabsetzung, während die Blutdiastase der akuten Form sich zur Steigerung neigt.

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Absorption of Crystal Violet by Candida Albicans

(A Method Proposed for Pathogenic Discrimination of Candida Albicans. II)

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In a previous report,[1] we have proved the frank difference in heat resistance of strains of Candida albicans obtained from foci of patients and other pathological materials, and those isolated from faeces of healthy persons, and proposed the utility of this difference for determining the pathogenicity of a Candida albicans strain. However, the small number of the strains experimented upon gave us some doubt on the accuracy of our conclusion. Acting on the announcement of Higuchi[2] that the toxicity of the strains of saccharomyces, myceloblastanon, cryptococcus and mycoderma can be determined by their capacity of absorbing dyes as a clue, we have followed up this phenomenon with Candida albicans as subjects, at the same time trying to reconfirm our previous conclusion.

Experimental

Materials: The same strains cited in the previous report[1] were used again. Crystal violet (Takeda) was selected to represent the dyes belonging to triphenylmethan and eosin A. G. (G. Grubler) to represent those of xanthen.

Method: To Sabouraud's glucose agar media, crystal violet was added in the ratios of 2×10^{-5}, 1×10^{-5}, $10/2\times10^{-6}$, $10/3\times10^{-6}$ and $10/4\times10^{-6}$, and eosin in the ratios of 1×10^{-3}, 2×10^{-4}, 1×10^{-4}, 2×10^{-5} and 1×10^{-5}, and their surfaces formed in slants. Upon these surfaces, candida cultivated for 7–10 days at 27°C was smeared in double parallel lines, the media then kept in an incubator for three days at 37°C and exposed to room temperature. The cultures were observed on the 5th, the 10th, the 20th and 30th days thereafter; the colonies that had clearly absorbed the dye and become stained were marked (+) or (++) according to the degree of coloration, those that showed no trace of absorption, that is, when no pigmentation appeared at all, as (−), and those that showed

only indecisive traces of absorption as (±).

Results: The data obtained were as follows and were showed in Table I en bloc.

1) In the cases when crystal violet was added to the extent of 2×10^{-5} and 1×10^{-5}, no strain was able to grow. When crystal violet content was $10/2\times10^{-6}$, Candida albicans No. 1–6, as well as No. 65, 67, 85 and 107, absorbed the dye in 5 days without exception, while No. 13 stained distinctly in 10 days and No. 47 in 30 days, No. 7078 less distinctly in 10 days and No. 7491 in 30 days. At $10/3\times10^{-6}$, No. 1–6 distinctly absorbed the dye in 5 days, while No. 13, 47, 65, 67, 85 and 107 showed no trace of staining at all or stained only indistinctly. No. 7078 and 7491 behaved in a similar manner. At the dilution of $10/4\times10^{-6}$, all strains were to be marked (−) or (±).

Of other species of candida, at the dilution of $10/2\times10^{-6}$ C. Guilliermondi was (+) in five days, (±) on the 10th and the 20th days and again (+) on the 30th day, C. tropicalis remained only indistinctly stained on the last 30th day, while C. parakrusei, C. stellatoidea and C. pseudotropicalis began distinctly to absorb the dye in 5 days. At $10/3\times10^{-6}$, C. Guilliermondi became distinctly stained in 20 days, but C. stellatoidea remained indecisive till the 30th day, while C. parakrusei and C. pseudotropicalis were already distinctly stained on the 5th day. At $10/4\times10^{-6}$, C. Guilliermondi and C. parakrusei became distinctly stained in 5–10

TABLE I

Cristal Violet Resorption of Candida Genus

Dilution of dye		$10/2\times10^{-6}$				$10/3\times10^{-6}$				$10/4\times10^{-6}$			
Days after transplantation		5	10	20	30	5	10	20	30	5	10	20	30
Candida albicans	No. 1	+	+	+	+	+	++	++	++	++	±	±	±
	2	+	+	+	+	+	++	++	+	++	±	±	±
	3	+	+	+	+	+	+	+	++	+	+	±	±
	4	+	+	+	+	+	+	+	+	±	±	−	−
	5	+	+	+	+	+	+	+	+	++	±	±	±
	6	+	+	+	+	+	+	+	+	++	±	±	±
	13	±	+	+	+	−	−	−	−	±	±	±	±
	47	−	±	±	+	−	−	−	−	−	−	−	−
	65	+	+	+	+	−	−	±	±	±	±	±	±
	67	+	+	+	+	−	−	−	−	−	−	±	±
	85	+	+	+	+	−	−	−	±	−	−	−	−
	107	+	+	+	+	−	−	−	±	−	−	±	±
	7078	±	+	+	+	±	−	−	−	−	−	±	±
	7491	±	±	±	+	−	−	−	−	−	−	−	−
C. Guilliermondi		+	±	±	+	±	±	+	+	+	+	−	−
C. tropicalis		−	±	±	±	−	−	±	±	−	−	−	−
C. parakrusei		+	+	+	+	+	+	+	+	+	+	−	−
C. stellatoidea		+	+	+	+	−	−	±	±	−	−	−	−
C. pseudotropicalis		+	+	+	+	+	+	+	+	+	+	+	+

days, but exceptionally lost their once absorbed dye in 20–30 days, while C. tropicalis and stellatoidea refused to absorb dye throughout, and C. pseudotropicalis became stained distinctly in 5 days.

2) Eosin was absorbed by all the strains without difference, in the range of our experiments given above.

Summary and Discussion

Epstein[3] seems to have been the first in taking up the dye absorption of microbes for determining their toxicity. Higuchi[2] has tried the method on eumycetes, but not on microbes of the genus Candida, so we have proceeded with the experiment on candida applying Higuchi's method. Summarizing the results of our experiments on the dye absorption of different strains of candida, we find the following: When Candida albicans strains are cultivated on Sabouraud's glucose agar media to which crystal violet has been added in the dilution of $10/3\times10^{-6}$, the strains that had been isolated from disease foci and other pathological materials are distinctly observed to absorb the dye, while those isolated from faeces of persons in normal health do not show any absorption. Such findings clearly indicate the possibility of determining the pathogenicity of C. albicans strains by dye absorption, inasmuch as the results are in good agreement with the outcome of our previous experiments[1] on heat resistance. On the other hand, it must be noted that the results were quite indifferent when the crystal violet content was higher than $10/2\times10^{-6}$ or lower than $10/4\times10^{-6}$ inclusive, so that it is apparent that strict attention must be exercised in maintaining the proper dilution of the dye.

Of the candida strains shared from Duke University, the two strains of Candida albicans showed the same disability to absorb the dye at $10/3\times10^{-6}$, as those of our known to have been isolated from faeces of healthy persons, a result concurring with that of heat resistance test. As to the other strains, we could not find a definite relation between the results of the dye absorption and heat resistance tests.

In our former report[1] we have suggested the analogous relation of heat resistance and pathogenicity observable in strains of candida other than albicans, but we have been taught that our deduction was untenable, by the above discrepancies in the results of the two studies, and also by a description of a predecessor[4] which has come to hand after the publication of our previous report.

The addition of eosin in the range of dilution of 1×10^{-3} to 1×10^{-5} caused no difference, as all the strains equally absorbed the dye, and consequently, no correlation of the absorption and the heat resistance could be traced.

In conclusion, we must mention with appreciation that the above study has been made possible by the allocation of Scientific Research Fund by the Education Ministry.

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Biochemical Studies on Carbohydrates

CLXXI. A Glucidamin[1] and a Neutral Mucopolysaccharide from Pig Spleen

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In a foregoing work[2] the fractions of the extract with 10% NaCl of pig spleens (fresh) proved Neuberg & Saneyoshi- and Goldschmiedt-negative.[3] Furthermore, judging from the faintly positive or negative results of Molisch test, they comprised only small amounts of glucidamin and neutral mucopolysaccharide at most. The tissue residue after the exhaustion showed also no positive reactions for hexuronic acid so that pig spleen is considered to be almost, if not perfectly, devoid of hexuronic acid-containing polysaccharide, while it gave the positive Molisch in addition to the positive biuret, suggesting the presence of a glucidamin or glucidamins and/or a mucopolysaccharide or mucopolysaccharides composed of non-hexuronic acid sugars. The writer undertook to separate these components from materials corresponding to the extracted tissue residue in the former work. The preparation procedure and the properties of the products will be described in the experimental part.

Experimental

Preparation Procedure

The preparation was carried out during winter in an unheated room (−5 — +5°C).

Fresh pig spleens freed from adipose tissues and blood vessels were extracted three times with 3 volumes of 10% NaCl at below 5°C as already described[2]. The residue was roughly washed by three repetitions of short agitation with 3 volumes of ice-cooled water, squeezed and then covered under 4.5 volumes of 91% ethanol for 5 days, followed by treatment in a Faust apparatus. 590 g. was the dry substance thus obtained from 96 spleens. It showed strong biuret, moderate Molisch and slight indirect-Osaki-Turumi reaction, but none of Bial-Mejbaum, Neuberg-

Saneyoshi, Goldschmiedt, direct-Osaki-Turumi and Hiyama reaction.

The material was pulverized in an iron mortar and divided into two equal portions for successive runs. Namely, 295 g. of the light brown powder were taken in one run, and shaken with 5 volumes of water, employing a few drops of toluene as a preservative. Centrifuged. To the light brown, turbid supernatant was added 50% acetic acid to pH 3.2. The bulky precipitate, which was almost Molisch- and indirect-Osaki-Turumi-negative,[4] was rejected. The mother fluid was dialyzed for 3 days, distilled under reduced pressure to dryness at 35°C and after transferring to a dish by means of ethanol, dried in vacuo over $CaCl_2$. The tissue residue was treated twice more in a like manner, until it hardly gave the positive Molisch reaction. The solutions hereby obtained were treated as the corresponding solution above and the acid-soluble products were combined with the one above. 725 mg. in total of a yellow powder were given—**Fr. A.** Molisch and biuret ##. Hiyama and indirect-Osaki-Turumi +, phosphorus ±. Neuberg-Saneyoshi, Goldschmiedt and direct-Osaki-Turumi −.

Next, the balance of Fr. A (700 mg.) were taken up in 30 cc. of water, and after removal of a small insoluble part, alkalized to pH 9.0 with 0.5 *N* NaOH. A brownish deposit occurred, which was centrifuged, washed with a very dilute NaOH of pH 9.0 and dried. It gave strong biuret but only faint Molisch[5] so that it was discarded. The mother fluid of the deposit gave no precipitate anymore on acidification to pH 3.2 with acetic acid but a tiny precipitate on further acidification to pH 1.0 with HCl (10%). After centrifuging off of this precipitate (biuret ##, Molisch −), the supernatant was added to with NaOH (1 *N*) carefully in drops to pH 9.0, whereat again a tiny precipitate occurred. The precipitate here (It weighed 4 mg. when dried. Biuret ##, Molisch ±)[6] was removed. The schift of pH to 1.0 was repeated and a trace of precipitate was again rejected. By again moving back the pH to 9.0, no more precipitate appeared. Finally the fluid was adjusted to neutral. Tested on small parts, it gave no precipitate by barium acetate (20%), but turned somewhat cloudy on addition of $CuCl_2$ (2 *N*) to pH 4.0. Therefore, the whole remaining part was freed from the cupric chloride-precipitable (After removing copper by HCl-containing alcohol, it weighed 6 mg. and gave strong biuret but only faint Molisch),[7] and dialyzed for 3 days. The slightly blue-colored fluid was distilled in vacuo to a few cc. and an excess of abs. alcohol was added. The deposit occurring was washed with HCl-containing alcohol (a mixture of 99 volumes of 95% alcohol and 1 volume of 15% HCl) and alcohol in sequence and dried. A yellowish powder weighing 510 mg. was yielded—**Fr. B.** Qualitative tests: biuret ##, Hiyama −, Molisch ##, indirect-Osaki-Turumi +, Neuberg-Saneyoshi and Goldschmiedt −, direct-Osaki-Turumi

−, P −.

Fr. B was subdivided by means of $(NH_4)_2SO_4$ as follows. 490 mg. of it were dissolved in 30 cc. of water, saturated with ammonium sulfate and after standing overnight, centrifuged. The centrifugate was reprecipitated from the same volume of water as above, and electrodialyzed as dissolved in 50 cc. of water for 60 hours, until no more SO_4^{--} and NH_4^+ were detected. Some deposit which had appeared during dialysis was filtered off and the filtrate was distilled to about 2.5 cc. in vacuo. The condensate was precipitated with 10 volumes of abs. alcohol and 1 cc. of abs. alcohol saturated with sodium acetate, washed with alcohol and with ether and dried in a desiccator over H_2SO_4. 290 mg. of a white powder were given—**Fr. C** (crude Glucidamin). Biuret ⫩, Molisch ⫩, indirect-Osaki-Turumi +, direct-Osaki-Turumi −, Rosenthaler for methylpentose +, Bial-Mejbaum for pentose −. The two successive, ammonium sulfate-saturated supernatants were united and after dilution with 5 volumes of water, dialyzed for 4 days, vacuum-distilled at 35°C to about 150 cc. and electrodialyzed for 72 hours. The solution thus completely freed from the deproteinizing agent was concentrated further to about 1.5 cc. and precipitated with alcohol containing sodium acetate. The deposit was washed with alcohol and ether and dried similar to above. 90 mg. was the white powder given —**Fr. D** (crude Neutral Mucopolysaccharide). Molisch ⫩, indirect-Osaki-Turumi +, direct-Osaki-Turumi −, Rosenthaler for methylpentose +, Bial-Mejbaum for pentose −, biuret ±.

Fr. C showed an accessory boundary besides the main after prolonged electrophoresis at pH 7.8 (S. Fig. 1), and Fr. D appeared to be contaminated with a protein or proteins, because it was not perfectly biuret-negative. Therefore, those fractions were purified by the use of glacial acetic acid :—

a) 255 mg. of the crude glucidamin (Fr. C) were kneaded with 5 drops of water and 10 cc. of glacial acetic acid (98%) were added in portions, whereby slight turbidity occurred. Centrifuged. The yellowish transparent supernatant was distilled under reduced pressure at 20°C to about 2 cc. and 10 cc. of dry ether were added. The precipitate was washed with ether exhaustively and dried in vacuo over solid caustic soda. 215 mg. was the white powder obtained. After the second run of preparation were given 230 mg. of a powder of resembling appearance. The products were put together—**Fr. E** (**Glucidamin**).

b) To 60 mg. of Fr. D were added 2 drops of water and the paste was stirred up after addition of 5 cc. of 98% acetic acid and centrifuged. The clear supernatant with faint yellow hue was decanted out and the centrifugate was treated similar to above over again, using 1 drop of water and 5 cc. of the acetic acid. The centrifugate here was washed with 5 cc. of the acetic acid and then with many portions of ether and dried in vacuo

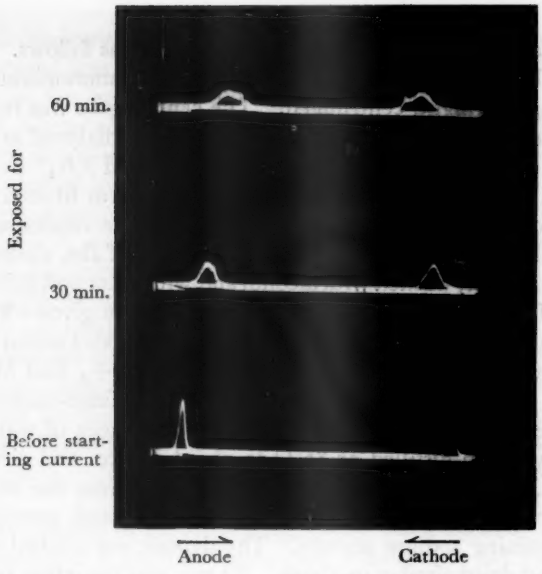

Fig. 1. Electrophoretic patterns of a 1.0% solution of Fr. C (crude Glucidamin) in phosphate buffer of pH 7.8 and I 0.29. Temp. of test 16°C; cur. 10 mA. Ascending limbs to the left.

over solid caustic soda. A white powder was produced. Crop 40 mg. From the two successive centrifuged supernatants were recovered only 4 mg. of a biuret-positive substance in a manner described in a), which was rejected. By the second run of preparation 65 mg. of the glacial acetic acid-insoluble were given. It was combined with the corresponding product above—**Fr. F (Neutral Mucopolysaccharide)**.

Properties of the Purified Preparations of Glucidamin and Mucopolysaccharide

The glucidamin

Electrophoresis. The product proved homogeneous, a single boundary being demonstrated after electrophoresis of the substance at pH 7.8. S. Fig. 2.

Solubility and precipitability. It was found transparently soluble in water of pH's from 1.0 to 9.0, 98% acetic acid, formamide, ethyleneglycol and 10% trichloroacetic acid but insoluble in ethanol and ether. Lead acetate, cupric chloride, barium acetate and baryta did not precipitate it from water. The glucidamin was nearly entirely precipitable from water

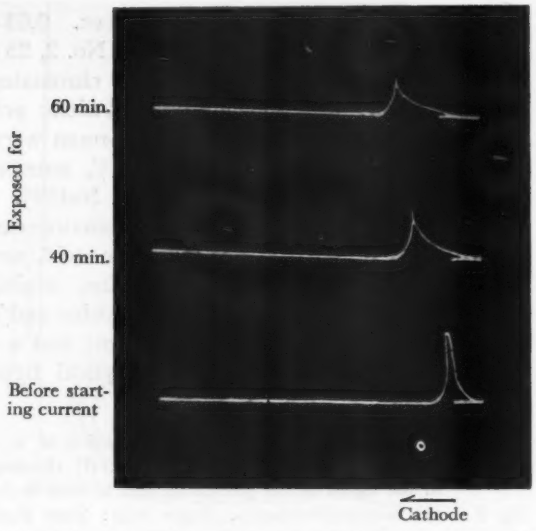

Fig. 2. Electrophoretic patterns of a 1.0% solution of Fr. E (Glucidamin) in phosphate buffer of pH 7.8 and I 0.29. Temp. of test 15°C; cur. 10 mA. Descending limbs alone are shown. Mobility $= \frac{0.75 \times 0.01021 \times 0.3}{0.01 \times 3600}$ $= 6.4\ cm^2 \cdot volt^{-1} \cdot sec^{-1} \cdot 10^5$.

by ammonium sulfate at full saturation but as much as a half by the same salt at half saturation and Na_2SO_3 at full saturation, and only slightly by Na_2SO_3 at half saturation and NaCl at full saturation.

Qualitative test-tube tests. Molisch ±, indirect-Osaki-Turumi +, Rosenthaler for methylpentose +, direct-Osaki-Turumi −, Bial-Mejbaum −, Neuberg-Saneyoshi and Goldschmiedt −, P −, biuret ±, Hiyama −, hydrolysable S −.

Paper partition chromatography. 1) Sugar components. The technique of Masamune & Yosizawa[8] was adapted, starting with 35 mg. of the preparation. The hydrolysate nearly freed from H_2SO_4 was condensed to about 0.15 cc. and 0.04 cc. was sampled. As is shown in Fig. 3(b), spots at the positions corresponding to chondrosamine, glucosamine, galactose, mannose and L-fucose were given in the chromatogram.

2) Amino acid components. The material taken for hydrolysis was 35 mg. It was refluxed at 100°C for 24 hours with 3.5 cc. of 6 *N* HCl. The hydrolysate and washing were evaporated together on a boiling water bath and the residue was further freed from the hydrochloric acid as far as possible by repeated evaporation with water. The final residue was aken up in 10 cc. of water (pH of the solution 5.0) and filtered, and the

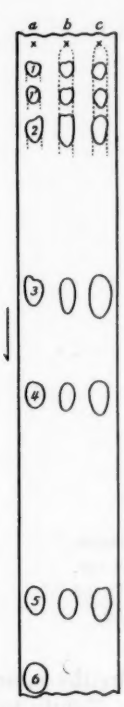

Fig. 3

Fig. 3. Diagrammatical reproduction of a *n*-butyl-acetate-acetic acid-ethanol-water (3:2:1:1) chromagogram of the sugars in the glucidamin and of those in the neutral mucopolysaccharide. Paper strip: Toyo Roshi filter paper No. 2, 7.5 cm. × 60 cm. Temp. and duration of test 20-22°C and 48 hrs. respectively. Indicator aniline hydrogen phthalate of Partridge.
a: reference run, b: Glucidamin, c: Neutral Mucopolysaccharide, 1 and 1′: chondrosamine sulfate, 2: glucosamine sulfate, 3: galactose, 4: mannose, 5: L-fucose, 6: rhamnose.

filtrate was concentrated to about 0.3 cc. 0.03 cc. of it was sampled on a Toyo Roshi filter paper No. 2, 25 cm. × 25 cm. square, to develop a two-dimensional chromatogram in the ascending way with solvents butanol-acetic acid-water (4 : 1 : 2) and 80% phenol (In the bell-room were placed two dishes, one containing 2–3 cc. of 1% ammonia and the other containing a spoonful of solid NaCN). In Fig. 4 is diagrammatically reproduced the chromatogram, which possessed spots of aspartic and glutamic acid, serine, glycine, hexosamine, alanine, lysine, histidine, arginine, proline, valine and/or methionine, tyrosine, leucine and/or isoleucine, phenylalanine, threonine (a weak spot) and a peptide.

Quantitative analyses. The analytical figures obtained are embodied in Table I.

Optical rotation in water. $[\alpha]_D^{17} = \frac{-0.236 \times 1}{0.0105 \times 1} = -22.5°$.

Ultraviolet absorption. A 0.025% solution in water of the substance (pH 6.0) was examined in a Hitachi EPB-U type spectrophotometer. Cell 1 cm. thick. Curve A in Fig. 5 was constructed from the extinctions measured. The weak absorption between wave-lengths 250 and 310 mμ with the maximum at 270–280 mμ might be due to the cyclic amino acids.

Group specificity. The procedure of examination was the one usual to us. A and B sera having titre 320 and an eel serum having titre 1600 were all diluted to titre 16 for the assay. As seen in Table II, the glucidamin proved Group A specific.

The neutral mucopolysaccharide

Electrophoresis. One boundary only was demonstrated after electrophoresis at pH 7.8 of the preparation as in the case of the glucidamin. See Fig. 6.

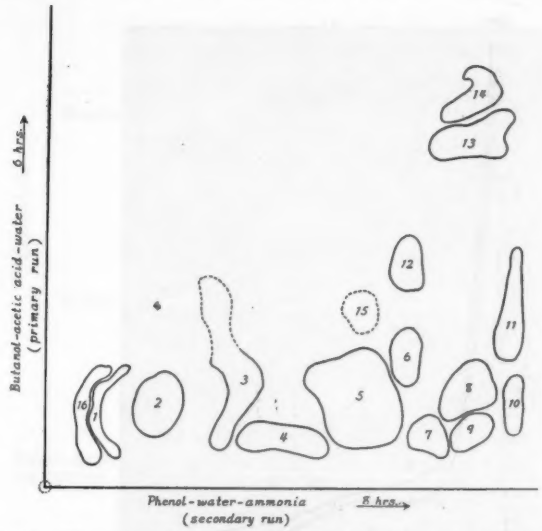

Fig. 4. Diagrammatical reproduction of the amino acid chromatogram of the glucidamin. Temp. of test 20–22°C. Indicator 0.15% ninhydrin in water-sat. butanol.

1: aspartic acid, 2: glutamic acid, 3: serine, 4: glycine, 5: hexosamine, 6: alanine, 7: lysine, 8: histidine, 9: arginine, 10: proline, 11: valine and/or methionine, 12: tyrosine, 13: leucine and/or isoleucine, 14: phenylalanine, 15: threonine, 16: a peptide.

TABLE I

Composition of the Glucidamin

Analysis	In per cent	In equivalents per equivalent weight
N*	11.9	
Hexosamines as glucosamine†	13.0	1.0
Galactose‡	9.4	0.72
Mannose‡	3.2	0.24
L-Fucose§	3.6	0.30
Ash//	0.9	

* Micro Kjeldahl, † Blix[9], ‡ Masamune & Ogawa[10], § Masamune & Ogawa[11], // Pregl method without use of H_2SO_4.

Solubility and precipitability. Clearly soluble in water at various pH's from 1.0 to 9.0, formamide, ethyleneglycol, 10% trichloroacetic acid and the sat. $(NH_4)_2SO_4$. Insoluble in 98% acetic acid, ethanol and ether. Non-precipitable by cupric chloride, barium acetate and lead acetate.

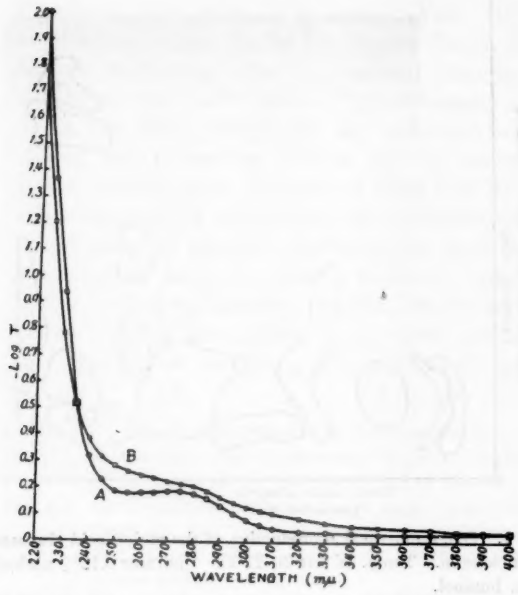

Fig. 5. Ultraviolet absorption spectra of a 0.025% solution in water of Glucidamin (Curve A) and of a 0.1% solution in water of Neutral Mucopolysaccharide (Curve B). Slit 1.22 mm/220 mμ.

TABLE II

Occurrence of Agglutination of A, B and O Erythrocytes by Human B and A Sera and an Eel Serum, treated with the Glucidamin, respectively

−: no agglutination, ±: faint aggl., +: slight aggl., ++: marked aggl., +++: strong aggl.

Group of red cells	Dilution of the substance (1:)											
	10^3	10^4	2×10^5	4×10^5	6×10^5	7×10^5	8×10^5	10^6	2×10^6	4×10^6	8×10^6	∞
A	−	−	−	−	−	±	+	+	++	++	+++	+++
B	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
O	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Qualitative test-tube tests. Molisch +++, indirect-Osaki-Turumi +, direct-Osaki-Turumi −, Rosenthaler for methylpentose +, Bial-Mejbaum −, Neuberg-Saneyoshi −, Goldschmiedt −, hydrolysable S −, biuret −, Hiyama −, P −, iodine test for glycogen −.

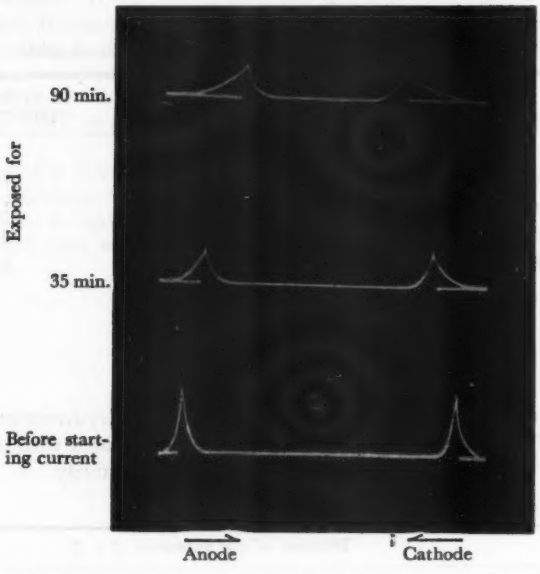

Fig. 6. Electrophoretic patterns of a 1% solution of the neutral mucopolysaccharide in phosphate buffer of pH 7.8 and I 0.29. Temp. of test 15.5°C; cur. 10 mA. Ascending limbs to the left. Mobility of the descending boundary $=\frac{0.8\times 0.012450\times 0.3}{0.01\times 5400}=5.5$ cm^2·volt^{-1}·sec^{-1}·10^5.

Paper partition chromatography of the sugar components. 12 mg. of the substance were taken for the chromatography according to Masamune & Yosizawa[8]. The hydrolysate, freed from most of H_2SO_4 and humin, was condensed to about 0.15 cc., and 0.04 cc. of it was sampled on the same paper sliver that was spotted with the corresponding condensate above to develop the chromatograms in parallel. Spots of the same sugars as those in the glucidamin were detected (Fig. 3(c)).

Quantitative analyses. Table III shows the analytical figures obtained.

Optical rotation in water. $[\alpha]_D^{\prime\prime}=\frac{-0.169\times 1}{0.0103\times 1}=-16.4°$.

Ultraviolet absorption. A 0.1% solution was examined as above. See Fig. 5 (Curve B). Whether the faint absorption between 270 and 290 mμ is of meaning or not will be settled by a further study.

Group activity. Tested similar to above with the same sera. The mucopolysaccharide appeared not to possess this serological property. See Table IV.

TABLE III

Composition of the Neutral Mucopolysaccharide

Analysis	In per cent	In equivalents per equivalent weight
N*	2.8	
Hexosamines as glucosamine†	27.4	1.0
Galactose‡	27.9	1.0
Mannose‡	9.0	0.33
L-Fucose§	10.3	0.41
Ash//	0.8	

*, †, ‡, §, // Signify the same as in Table I.

TABLE IV

Occurrence of Agglutination of A, B and O Erythrocytes by Human B and A Sera and an Eel Serum, treated with the Neutral Mucopolysaccharide, respectively

+, ++ and +++ signify the same as in Table II.

Group of red cells	Dilution of the substance (1 :)					
	2×10^2	4×10^2	8×10^2	1.6×10^3	3.2×10^3	∞
A	+	++	+++	+++	+++	+++
B	++	++	+++	+++	+++	+++
O	++	++	+++	+++	+++	+++

SUMMARY

A glucidamin and a neutral mucopolysaccharide were separated in an electrophoretically homogeneous state and investigated physically, chemically and serologically.

Through the Grant Committee for Scientific Researches was given a grant from the Education Department, which enabled us to carry out this work.

H. Masamune.

References and Notes

1) Cf. Masamune, Tohoku J. Exp. Med., 1949, **50**, 107; Masamune, Yosizawa & Maki *ibid.*, 1951, **53**, 237 (Footnote on p. 237); Masamune, Seitai no Kagaku (Jap.), 1952, **3**, 234 (See the explanation of Fig. 1).

2) Ogawa, Tohoku J. Exp. Med., 1952, **56**, 147.

3) In the 10th line from the bottom on the third page (p. 149) of that article, three words " Neuberg-Saneyoshi and Goldschmiedt " were missed between the words " Molisch " and " negative ", namely that line are to be read " markedly positive but Molisch, Neuberg-Saneyoshi and Goldschmiedt negative ". H. Masamune.

4) Further tests: It was Neuberg-Saneyoshi- and Goldschmiedt-negative but strongly biuret- and slightly Hiyama-positive, and contained no more than a trace of phosphorus.

5) It amounted to 25 mg. Further qualitative tests resulted: Hiyama +, indirect-Osaki-Turumi ±, direct-Osaki-Turumi −, P a trace.

6) Further tests: direct-Osaki-Turumi −, indirect-Osaki-Turumi ±, Hiyama +.

7) Further tests: direct-Osaki-Turumi −, indirect-Osaki-Turumi ±, P a trace, Hiyama −.

8) Masamune & Yosizawa, Tohoku J. Exp. Med., 1953, **59**, 1.

9) Blix, Acta chem. Scand., 1948, **2**, 467.

10) Masamune & Ogawa, Tohoku J. Exp. Med., 1954, **60**, 11.

11) *Idem, ibid.*, 1954, **60**, 33.

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Nucleic Acids

IV. DNA of Pig Spleen in High-associated State (A New Method of Preparation of DNA)

By

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In 1948, Petermann & Lamb[1] pointed to the occurrence of enzymic degradation of beef spleen DNA when it is extracted with 1 *M* NaCl, and recommended sodium citrate for inhibiting the action of desoxyribonuclease an enzyme concerned. According to Greenstein,[2] mouse spleen surpasses other organs of this animal in thymonucleodepolymerase force, being nearly six times as active as calf thymus. The writer found that pig spleen contains also no small amount of the enzyme, and was compelled to resort to an appropriate means of avoiding the depolymerization. Fortunately 3 *M* NaCl proved superior to 1 *M* NaCl containing 0.01 *M* sodium citrate of Petermann & Lamb not only in this respect, as will be illustrated below, but also in facilitating the extraction of the high-polymeric DNA.

To prepare the DNA from the DNP once separated, the former was precipitated from the solution of the latter saturated with NaCl by addition of 90% alcohol to 54% (Cf. Hammarsten[3]). DNA is carried down by alcohol at the concentration above and DNP by alcohol at 60% selectively. Fractionated precipitation of the acid with alcohol from the protein solution half saturated with NaCl (neutral) required many repetitions probably due to less dissociation of the protein.

In literature, sodium salt of beef spleen DNA of Petermann & Lamb[1] analyzed 15.1% N and 9.3% P, and that of Chargaff *et al.*[3a] 14.7% N and 8.8% P.

Experimental

Preparation Procedure of the DNA (Na-salt)

A typical example will be given.

The whole procedure was carried out in an unheated room in winter (−4−+4°C).

Preliminary treatment of tissue. Twelve fresh pig spleens cooled in ice were quickly brought from the slaughter house, freed from the adhering tissue, and after cutting into small pieces, roughly washed with 1.5 volumes of physiological saline twice, followed by grinding in a meat-chopper. 1300 g. of a brei were obtained. It was agitated with 1.5 volumes of 0.14 *M* NaCl for 2 hours and stood for a while, and the supernatant fluid was decanted out. The manipulation was repeated three times more. The tissue residue was then centrifuged, covered under two changes of 1.5 volumes of the saline solution overnight and for 6 hours and further under 1.5 volumes of 0.2 *M* NaCl[4] for 2 hours in succession with occasional shaking. The final centrifuged supernatant gave no precipitate by trichloroacetic acid.

Separation of DNA. The centrifugate was stirred up with 2, 1, 1 and 1 volumes of 3 *M* NaCl for 12, 3, 3 and 12 hours respectively in sequence. The fourth solution gave almost no fibrous precipitate by alcohol and was rejected. The first three (light orange-colored, somewhat turbid and viscous; pH about 6.8) were each filtered through two sheets of Toyo Roshi filter paper No. 2 laid one over the other under suction. To the clear filtrates were added 2 volumes of 90% ethanol, and the fibrous DNP precipitates were taken out after winding round a glass rod and immersed in 90% alcohol. The products from the three filtrates were united—**Fr. A** (DNP). Molisch faintly positive, Dische (for desoxypentose) and biuret strongly positive.

2 volumes of 90% ethanol were found to carry down nearly all of DNA in the form of DNP from the solution. On addition of an additional small volume of the alcohol to the separated mother fluid, a voluminous flocculent precipitate appeared together with a tiny fibrous, and on addition of more of the alcohol, NaCl precipitated in addition. On the other hand, dilution of the filtrate to 0.14 *M* NaCl caused precipitation of fibrous DNP, but the amount of the deposit was much less than that by 2 volumes of 90% alcohol.

Fr. A was squeezed and stirred up with portions of 10% NaCl reaching 3200 cc. in total, and thereafter the insoluble part was discarded. The solutions united (high-viscous) were saturated with NaCl and after placing in an ice chest for 5 days with occasional agitation, filtered under suction as above. The filtrate was added to with 1.5 volumes of 90% ethanol. The fibrous precipitate here (Molisch negative; biuret faintly positive) was also separated by means of a glass rod as above.

More than 1.5 volumes of the alcohol gives a flocculent precipitate besides.

Then it was taken up in 500 cc. of water and 1.5 volumes of 90% alcohol were added. The precipitate here which was also fibrous was redissolved and reprecipitated 5 more times, when it showed no biuret reaction and could be precipitated from its solution by the alcohol with

addition of a little sodium acetate only. The final deposit was washed with 54% alcohol, squeezed and freeze-dried. Yield 135 mg.

Properties of the Product

It gave no protein gel by Sevag procedure with a chloroform-octylalcohol (8 : 1) mixture, and gave two boundaries moving and stationary after electrophoresis at pH 7.8 (Fig. 1) but only the moving when electrodialyzed in advance, indicating that the stationary is ascribable to salt effect. It formed an extremely viscous solution (transparent) by shaking with water,

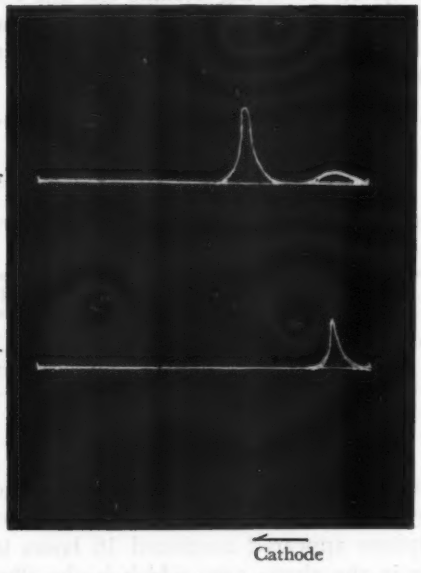

Fig. 1. Electrophoresis pattern of a 0.25% solution of the pig spleen DNA (Na-salt) in phosphate buffer of pH 7.8 and I 0.29. Temp. of test 10°C; cur. 13 mA, exposure 30 minutes. Mobility of the moving boundary= $\frac{1.3\times0.01062\times0.3}{0.013\times1800}=17.7$ cm^2·volt^{-1}·sec^{-1}·10^5. The descending limb alone is shown.

and a solution more concentrated than 0.25% was not subject to electrophoresis. Molisch negative, Dische (for desoxypentose) and Schiff (for aldehyde) intensively positive, Bial-Mejbaum faintly positive, Goldschmiedt and Neuberg-Saneyoshi negative. N (micro Kjeldahl) 15.7%; P (modified Plimmer) 9.1%; N/P 1.73; ash (Pregl without use of H_2SO_4) 22.7%.

Experiment regarding the Anti-DNAase Activity of NaCl

Six 100 g. portions of a pig spleen brei prepared as above were agitated with 300 cc. of 1 *M* NaCl, 1 *M* NaCl containing 0.01 *M* sodium citrate, 2 *M* NaCl, 2 *M* NaCl containing 0.01 *M* sodium citrate, 3 *M* NaCl or 3 *M* NaCl containing 0.01 *M* sodium citrate for 24 hours at below 4°C. The mixtures were subsequently incubated at 35°C together with 0.5 cc. of toluene, and from each, 5 cc. portions were taken out at intervals of 2 hours and centrifuged. The supernatant fluids were diluted with various quantities of water to 0.14 *M* NaCl. In all of the mixtures the amount of fibrous precipitate occurring became less and less with the course of incubation, and finally no fibrous precipitate but a small amount of the flocculent came out after the duration of time varying according to the mixture as shown in Table I.

The fibrous precipitates were Dische (for desoxypentose)-positive and nearly Molisch negative.

TABLE I

Time (hrs.) of Incubation till Cease of Occurrence of Fibrous Precipitate

Precipitation was caused with	Molarity of NaCl (above) and citrate (below) in the solution with which the brei was incubated					
	1 0	1 0.01	2 0	2 0.01	3 0	3 0.01
H_2O	16	30	28	32	32	32
Alcohol	32	46	44	48	48	48

In another run, dilution with water of the incubated mixtures was substituted by addition of 2 volumes of 90% alcohol. The time in which fibrous precipitate appeared continued 16 hours longer than the corresponding time in the above case, which is also illustrated in Table I.

The figures in the table indicate that the more the concentration of NaCl in the solution is, the more the depolymerase activity is inhibited, and that the citrate effect is no more witnessed when the concentration of NaCl is raised to 3 *M*.

SUMMARY

DNA of pig spleen was separated in a high-associated state by an easy method. The product comprised 15.7% N, 9.1% P and 22.7% ash.

Through the Grant Committee for Scientific Researches was given a grant from the Education Department in aid to us. H. Masamune.

References and Notes

1) Petermann & Lamb, J. Biol. Chem., 1948, **176**, 685.

2) Greenstein, J. Nat. Cancer Inst., 1941, **2**, 357.

3) Hammarsten, Bioch. Z., 1924, **144**, 388 (Footnote). He did not mention the percentage of the alcohol used, only describing " Es " (the solution in the sat. NaCl) " wurde in dünnem Strahl in $1^1/_2$ Vol. Alkohol gegossen.... ".

3a) Chargaff, Vischer, Doniger, Green & Misani, J. Biol. Chem., 1949, **177**, 405.

4) When washing with 0.2 *M* NaCl here was omitted, the final DNA-product was contaminated with some PNA.

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Biochemical Studies on Carbohydrates

CLXXII. Comparison of A and O Group Substances from Pig Stomach Mucus

By

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Group substances were separated one from each of the batches of Group A and Group O pig stomach mucus after splitting the mucus with NaOH at 0–3°C, as described in a previous article[1]. The preparations gave resembling electrophoretic patterns comprising two boundaries and proved also very similar regarding composition excepting L-fucose content. The two group substances showed distinct difference of optical rotation as already often met in corresponding pairs of group carbohydrates[1] prepared each from one tissue source by one and the same procedure. The divergency was still retained after HIO_4-oxidation and also after acid hydrolysis of the substances. Hence, its cause was considered to be sought to components or partial structures other than those attacked by these agents. Ultraviolet absorption of the preparations was examined besides.

Experimental

Material

The mucus of pig stomachs were separately dehydrated with alcohol, and 10 mg. each of the dry powders were swollen in 2.5 cc. of 0.01 *N* NaOH overnight and added to with 2.5 cc. of 1.8% saline solution, followed by progressive dilution with physiological saline. Group A activity was measured with normal B serum diluted to titre 16 and Group O activity with an eel serum diluted also to titre 16. Of 500 stomachs examined, 7 possessed Group A mucus (Group A active dilution $>10^5\times$, Group O active dilution $<10^3\times$), 32 Group O mucus (Group A active dilution $<5\times10^2\times$, Group O active dilution $>10^4\times$) and the others (461) both Group A and Group O active mucus.

Preparation of the Group Carbohydrates (polypeptide-polysaccharide complexes)

The stomach mucus dehydrated above was cleft at 0–3°C with 1 *N* NaOH and fractioned by following Masamune, Yosizawa & Miki[1] (See p. 152, 2) of the article). 150 and 180 mg. respectively of products were obtained from 3 g. each of the dry Group A and Group O materials. They were biuret-negative and Molisch- and indirect-Osaki-Turumi-positive, and will be referred to as Ch. A and Ch. O each to each.

Properties of the Group Carbohydrates

Serological. Ch. A was Group A active at a dilution of $1:2\times10^7$, but Group O active only at dilutions of $1:10^3$, of $1:2\times10^3$ and of $1:10^2$ respectively when an anti-O-erythrocytes immune serum, a normal anti-O goat serum and an eel serum were employed. The corresponding dilutions of Ch. O were $1:2.5\times10^2$, and $1:6.2\times10^4$, $1:1.24\times10^5$ and $1:6.2\times10^4$. The both substances were Group B inactive even at a dilution of $1:10^2$. See Table I.

Electrophoresis. After electrophoresis at pH 7.8 in a Tiselius apparatus, the both preparations gave two boundaries. The patterns resembled each other with respect to area, form and mobility of the boundaries (Fig. 1).

Ultraviolet absorption. 0.14% solutions in water not only of Chs. A and O but also of Ch. I_0 in the former work, which had been prepared likewise from mixed pig stomach mucus, (pH of the solutions 6.5) were examined in a Hitachi EPB-U type photoelectric spectrophotometer. The spectra are plotted in Fig. 2. A furthr study is requisite to settle if the faint

TABLE I

Group Specificity of Chs. A and O

A. Occurrence of agglutination of A and B erythrocytes by B and A Sera, treated with Ch. A or Ch. O, respectively

B and A sera both had titre 160 and were diluted 10-fold. − : no agglutination, + : slight aggl., ++ : marked aggl.

Group substance	Erythro-cytes	Dilution of the substances (1 :)										
		10^2	2.5×10^2	5×10^2	10^3	2×10^3	.....	10^7	2×10^7	4×10^7	8×10^7	Control*
Ch. A	A	−	−	−	−	−	.....	−	−	+	+	++
	B	+	++	++	++							++
Ch. O	A	−	−	+	++							
	B	+	++	++	++							

* Without substance.

B. Occurrence of agglutination of O erythrocytes by an anti-O erythrocyte immune serum, a goat serum serum or an eel serum treated with either Ch. A or Ch. O

The immune chicken serum had titre 1600 and after 20-fold dilution, absorbed with Ae erythrocytes. The serum absorbed had titre 16. The goat and eel sera had titres 160 and 1280 respectively, and were diluted to titre 16. ±: faint agglutination. Other notations signify the same as in **A** above.

Group substance	Serum	Dilution of substances (1 :)									
		10^3	2×10^3	4×10^3	8×10^3	1.6×10^4	3.2×10^4	6.4×10^4	1.28×10^5	2.56×10^5	Control*
Ch. A	Immune	−	+	+	+	++	++				++
	Goat-	−	−	+	+	+	++				++
	Eel-	−	±	+	+	+	++				++
Ch. O	Immune	−	−	−	−	−	−	−	+	+	
	Goat-	−	−	−	−	−	−	−	−	+	
	Eel-	−	−	−	−	−	−	−	±	+	

* Same as in **A**.

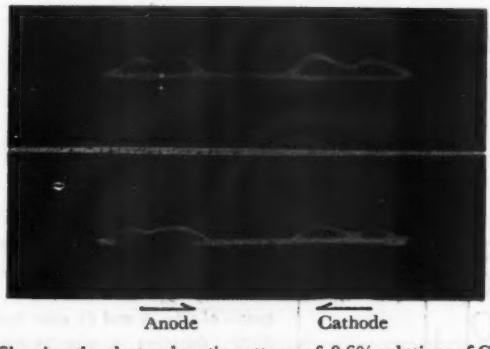

Fig. 1. Showing the electrophoretic patterns of 0.6% solutions of Chs. A (I) and O (II) in phosphate buffer, pH 7.8, I 0.145. Temp. 15°C; current 7 mA; exposure 60 minutes after starting current. Mobilities of the corresponding ascending boundaries were the same in the two substances. First moving boundaries 2.0 $cm^2 \cdot volt^{-1} \cdot sec^{-1} \cdot 10^5$; the second 7.2 $cm^2 \cdot volt^{-1} \cdot sec^{-1} \cdot 10^5$. Ascending limbs to the left.

absorption of Ch. A in the λ range from 247 to 265 mμ is significant.

Paper partition chromatography of their components. a) Sugars. The procedure described by Masamune & Yosizawa was applied without any modification, and the presence of chondrosamine, glucosamine, galactose and L-fucose in either of Chs. A and O was established. The chromatograms are reproduced diagrammatically in Fig. 3 (A and B).

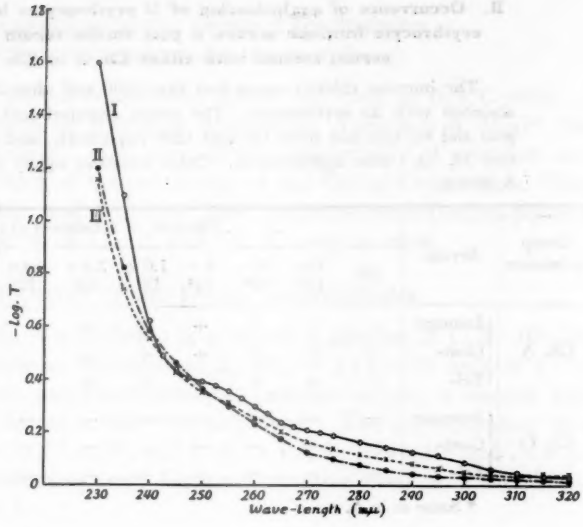

Fig. 2. Absorption spectra of 0.14% solutions of Chs. A (I), O (II) and I_0 (III) in water.

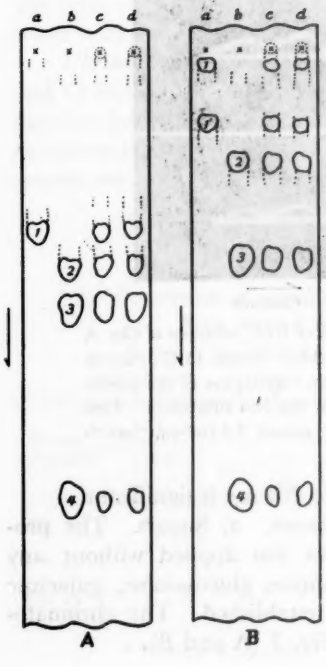

Fig. 3. Diagrammatical reproductions of butanol-pyridine-water (5 : 3 : 2) (A) and *n*-butylacetate-acetic acid-ethanol-water (3 : 2 : 1 : 1) (B) chromatograms of Chs. A and O after hydrolysis. Temp. 22-25°C; duration of test 24 hrs. Indicator aniline hydrogen phthalate of Partridge.

a, b: reference runs, c: Ch. A, d: Ch. O, 1, 1′: chondrosamine sulfate, 2: glucosamine sulfate, 3: galactose, 4: L-fucose.

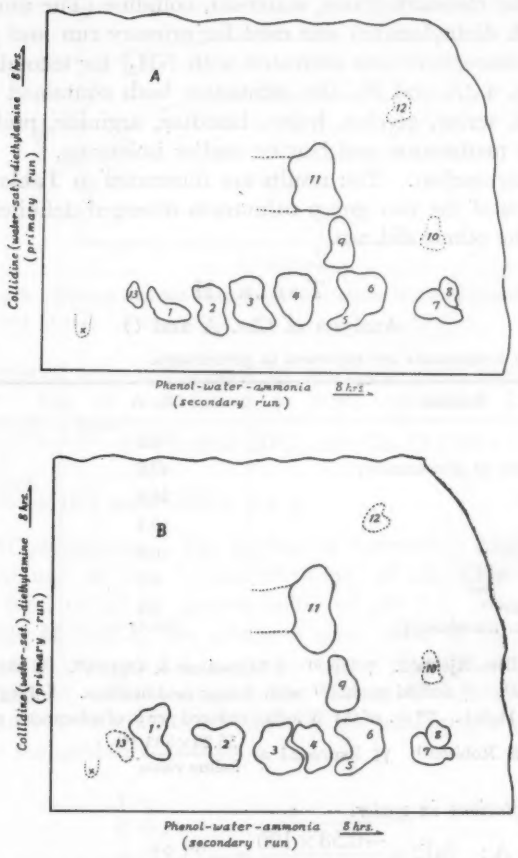

Fig. 4. Collidine(water-sat.)-diethylamine/phenol-water-ammonia chromatograms of Ch. A (A) and Ch. O (B) after hydrolysis. Temp. of test 23-25°C. Indicator 0.01% ninhydrin in butanol.

1 : aspartic acid, 2 : glutamic acid, 3 : serine, 4 : glycine, 5 : lysine, 6 : histidine, 7 : arginine, 8 : proline, 9 : alanine, 10 : valine and/or methionine, 11 : hexosamine, 12 : leucine and/or isoleucine, 13 : a probable peptide.

b) Amino acids. The method was what had been described by Masamune *et al.*[3] Of each of Chs. A and O, 40 mg. were taken and hydrolysis was continued for 24 hours. The evaporated residue of the hydrolysates, freed from HCl as far as possible, were taken up in 0.3 cc. of water and filtered, and a 0.025 cc. portion of the filtrates were sampled.

To develop the chromatograms, water-sat. collidine (The atmosphere was saturated with diethylamine) was used for primary run and phenol-water (4 : 1) (The atmosphere was saturated with NH_3) for secondary run. As shown in Fig. 4 (A and B), the substances both contained aspartic and glutamic acid, serine, glycine, lysine, histidine, arginine, proline, alanine, valine and/or methionine and leucine and/or isoleucine.

Quantitative analysis. The results are illustrated in Table II. The L-fucose contents of the two group substances diverged definitely from each other while the others did not.

TABLE II

Analyses of Chs. A and O

The components are expressed in percentages.

Analysis	Ch. A	Ch. O
N*	4.6	4.4
Hexosamines as glucosamine†	40.0	38.5
Galactose‡	34.8	33.4
L-Fucose§	10.5	13.5
Acetyl//	10.6	10.1
ash¶	0.5	1.5
Iodine value**	3.2	3.5
(m. w. calc. therefrom††)	(6614)	(6047)

* Micro Kjeldahl. † Blix.[5] ‡ Masamune & Ogawa[6]. § Masamune & Ogawa[7]. // Suzuki method[8] with minor modifications. ¶ Pregl without use of H_2SO_4. ** cc. of 0.1 *N* iodine reduced per g. of substance ; method, Macleod & Robison[9]. †† Expressed as $\frac{55.26\times 383}{\text{iodine value}}$.[10]

Optical rotations in water.

$$\text{Ch. A}: \quad [\alpha]_D^{14}=\frac{-0.58\times 100}{2.50\times 1}=-23.2°$$

$$\text{Ch. O}: \quad [\alpha]_D^{14}=\frac{-0.99\times 100}{2.656\times 1}=-37.3°$$

Consumptions of periodic acid and of sodium metaperiodate. a) HIO_4-treatment. To 1 cc. of a 1.83% and a 1.979% solution respectively of Chs. A and O was added 0.3 cc. of 0.4108 *M* HIO_4 and the optical rotation change at 14°C (room temperature) was followed up. After the lapse of 2 hours the rotations remained constant (Fig. 5). It is noteworthy that the specific rotations of the two group substances differed roughly to the same extent throughout the reaction. Then, another pair of reaction mixtures composed identically with those above each to each were prepared and stood at the same temperature as above for 2 hours and the amounts of the

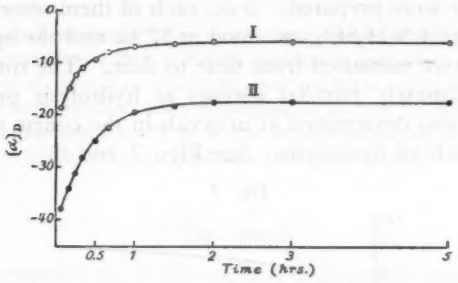

Fig. 5. Optical rotation change in the course of HIO_4-oxidation of Chs. A (I) and O (II). Temp. 14°C.

oxidant unreduced were determined by arsenious oxide method of Fleury & Lange.[3a] Ch. A was found to have consumed 2.84×10^{-3} ($=\frac{(0.1232-0.0712)\times10^{-3}}{18.3}\times10^{3}$) mole HIO_4 and Ch. O 2.90×10^{-3} ($=\frac{(0.1232-0.0658)\times10^{-3}}{19.79}\times10^{3}$) mole HIO_4 per g.

b) $NaIO_4$-treatment. The method of Aminoff & Morgan[4] was employed. 44.46 mg. of Ch. A and 49.46 mg. of Ch. O were separately dissolved in 6 cc. of 0.1 *M* acetate buffer of pH 5.0. After addition of 2 cc. of 0.3024 *M* $NaIO_4$, the mixtures were stood at 14°C (room temperature), and at intervals 0.5 cc. portions were pipetted out to titrate the unreduced oxidant. The Group A and Group O substances (preparations) used 3.0×10^{-3} and 3.1×10^{-3} mole of the oxidant per g. respectively. Fig. 6 shows the oxidation courses.

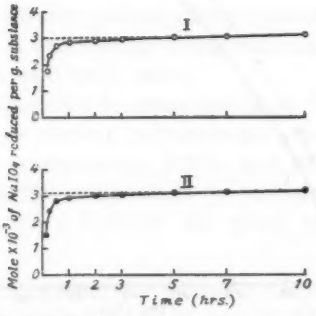

Fig. 6. $NaIO_4$ consumptions of Chs. A (I) and O (II) at pH 5.0 and 14°C as a function of time.

Hydrolysis with 2 N H_2SO_4 at 37°C. 1.83% Ch. A and 1.979% Ch. O

solutions in water were prepared. 5 cc. each of them were added to with an equal volume of 4 *N* H_2SO_4 and stood at 37°C, and the optical rotations of the mixtures were measured from time to time. The rotations changed following approximately parallel courses as hydrolysis proceeded. The iodine uses were also determined at intervals in the course of hydrolysis, to indicate the extents of hydrolysis. See Figs. 7 and 8.

Fig. 7

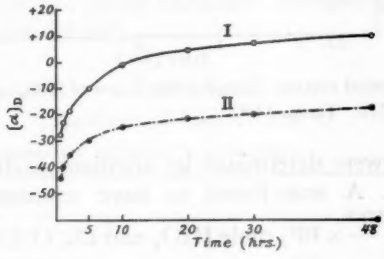

Fig. 8

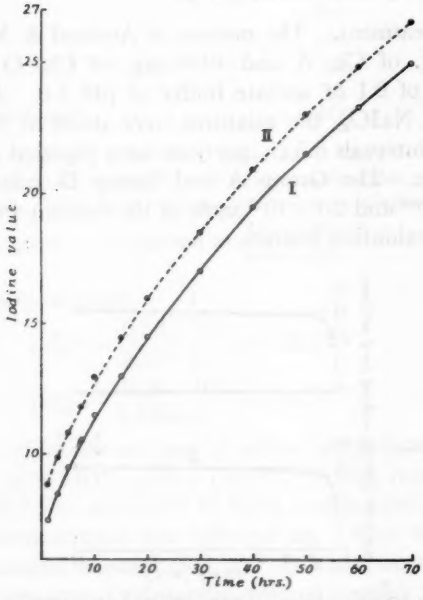

Fig. 7. Optical rotation change in the course of hydrolysis of Chs. A (I) and O (II) with 2 *N* H_2SO_4 at 37°C.

Fig. 8. Increase of iodine value of Chs. A (I) and O (II) on hydrolysis with 2 *N* H_2SO_4 at 37°C.

The method of estimating iodine use:—1 cc. portions of the mixtures taken out accurately were neutralized with 1 *N* NaOH (indicator a very dilute methylred solution in water), and 5 cc. of water, 2 cc. of 0.02 *N* iodine and 1.5 cc. of 5% Na_2CO_3 were added in turn. The mixtures here were stood in the dark at 20-21°C for 30 minutes, acidified immediately thereupon with 5 cc. (2 cc. at first and afterwards 3 cc. added) of 0.5 *N* H_2SO_4 and titrated with 0.005 *N* Na_2SO_3 (indicator 1% starch solution). The blank was carried out in parallel.

Discussion

1) The compositions of Chs. A and O mutually resemble in general. The higher L-fucose content of the former preparation may be of significance, but several specimens have to be prepared to derive a definite conclusion. The iodine values obtained can not be so accurate because of the so small iodine uses. Approximate accordingly as the minimum molecular weights calculated therefrom are, they reveal that the molecule of Ch. A is somewhat larger than that of Ch. O (It is assumed as if the preparations were single entities). It is probably due to a larger polypeptide, cleft together with polysaccharide by alkali, in Ch. A, although the analytical figures of total N in the two preparations differed scarcely. The resembling HIO_4 and $NaIO_4$ consumptions of the substances suggest similar structures of the polysaccharides in them.

2) That the substances showed parallel optical rotation changes when oxidized with HIO_4 as well as when hydrolyzed with acid might mean that the partial structures or components causing the rotation discrepancy are not attacked by those reagents.

Summary

1. Group carbohydrates (polypeptide-polysaccharide complexes) were separated from Group A and Group O pig stomach mucus, and their properties were compared with each other.

2. They differed not only in group specificity but also in optical rotation and possibly L-fucose content, but not in the other physical and chemical properties examined (composition, HIO_4- and $NaIO_4$-use and else).

3. It looked that the components or partial structures causing the optical rotatory discrepancy between the group carbohydrates are inert to HIO_4 and acid.

Through the Grant Committee for Scientific Researches was given a grant from the Education Department in aid to us. H. Masamune.

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2) Cf. Tiba, *ibid.*, 1949, **50**, 293; 1949, **51**, 309; Satoh, *ibid.*, 1949, **51**, 275, 281.

3) Masamune, Yosizawa & Masukawa, *ibid.*, 1953, **58**, 381.
3a) Fleury & Lange, J. pharmac. et chim., 1933, **17**, 107.
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7) Masamune & Ogawa, *ibid.*, 1954, **60**, 33.
8) Suzuki, J. Bioch. (Japan), 1938, **27**, 367.
9) Macleod & Robison, Bioch. J., 1929, **23**, 517.
10) Cf. Yosizawa, Tohoku J. Exp. Med., 1950, **52**, 151.

Note at proof-reading. This article has been read before the 26th Annual Meeting of the Japanese Biochemical Society, Sendai, April 25-27, 1954.

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Sensory Innervation of Urogenital Organs of Fourth Month Female Embryo

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Many studies, with excellent results, have been conducted and reported in the past on the subject with human adults as objective, but very few concerned with human embros, and as for studies on fourth month embryo, nothing has been heard of hitherto. Such being the case, I took up fourth month human female embryos as objective of my study and undertook to elucidate the innervation, especially the development of sensory fibres in their urinal system, in particular, in the distal part of the ureter, the bladder and the urethra, and in their genital system, in particular, in the uterus, the vagina, the vaginal vestibule, the labium minus and the clitoris.

The materials were fixed in 10% neutral formol for over half a year, then cut into a series of frozen sections approximately perpendicular to the general direction of the urethra and vagina, and stained by Seto's silver impregnation. These numerous preparations were subjected to a minute scrutiny. I wish to add that the preparations stained under this method have the characteristic of showing the pictures of nerve fibres with the better definition, the more the other various tissues are stained with beartiful distinctness.

Individual Views

Let me speak first briefly on the histological pictures of the urogenital organs in fourth month female embryo, prior to dilating upon the innervation thereof, because this is a subject with closest bearing upon the subject of innervation, but also because the subject has rarely been reported upon with any minuteness. As my impregnated preparations afford a rare chance of histological observation, I am sure a delineation of such observations will be fraught with a rich contribution to the elucidation of the subject.

The distal part of the ureter is markedly well developed in the fourth month embryo. The mucous membrane shows already some longitudinal folds. Its epithelium is especially interesting shown to consist of very thick transitional epithelium of 10 to 15 rows, not to be seen in adult. In collation with the fact that even the epithelium of the bladder consists of transitional epithelium of only 4 or 5 rows, it may be called a very peculiar phenomenon. Around the

epithelium, a tunica propria is formed, represented by connective tissue cells with an oval or spindle-form nucleus arranged circularly in a considerably dense alignment. The development of the tunica muscularis enclosing the tunica propira is as yet very poor, the cells showing a sporadic arrangement, but the development of the circular middle muscle layer is remarkable. Also the outer longitudinal muscle layer shows good development, especially in the inside walls. But what must be specially mentioned here is the fact that the smooth muscle fibres are very well developed, showing an image of muscle fibres scarcely inferior to those in adult in development.

The bladder has powerfully developed lengthwise folds, especially in its basis. The epithelium consists of transitional epithelium with 4 to 5 rows. The tunica propria is composed of connective tissue cells with an oval nucleus arranged sparsely and irregularly, its periphery going over to tunica submucosa provided with rather thick blood vessels. The connective tissue cells here are arranged more sparsely than in the tunica propria. The tunica muscularis shows only a very weak development as in the ureter, and nothing of the strength in adult bladder is observed. But the distinction between the inner longitudinal, middle circular and outer longitudinal layers is already marked, and the development of the muscle fibres themselves is as remarkable as in the ureter.

The urethra lies on the ventral side of the vagina and is connected directly with the vagina without any connective tissue septum. The proximal part of the urethra is provided with a broad urethral cavity and on its posterior wall, longitudinal folds are conspicuous. However, the urethra decreases in diameter as it approaches the outer orifice and the folds dwindle gradually information. The epithelium consists of stratified columnar epithelium of 5 or 6 rows. The tunica propria in the proximal part of the urethra is a comparatively broad layer, consisting of closely arranged connective tissue cells, containing many venous plexuses in the posterior wall, but on approaching the outer orifice, its development becomes gradually weaker, and near the orifice, only a few if any venous plexuses are found.

Around the tunica propria, muscle layers are found, which show strong development in fourth month fetus. In the proximal part of the urethra, there is an inner longitudinal and a much more strongly developed outer circular muscle layer, which is again covered with a weakly developed longitudinal muscle layer. But as the urethra proceeds toward the orifice, the inner and the outer muscle layers disappear and only the circular layer remains, around which some skeletal muscle fibres are added to the formation.

The uteurs is about 1.5 mm. wide, or of one fourth width of the bladder, containing a uterus cavity shaped as ∼ in cross-section. The epithelium is composed of long columnar cells with a long rod-like nucleus. These are so closely arranged that they seem to contain two or three rows but in reality there is only one row of them. No gland formation is found directed toward the surrounding tissue at this stage of growth.

The tissue surrounding the epithelium is represented by a considerably thick layer of very closely arranged connective tissue cells with an oval nucleus. These tissue cells do not all develop into tunica propria in the future, but the majority in the outer layer becomes the inner muscle layer, or the tunica submucosa later on. Only the inner layer becomes the tunica propria in the adult. Close on the outside of the former, a layer, of considerable thickness, running circularly, is formed of elongated cells containing a spindle-shaped nucleus. This layer shows already the nature of muscle fibres and represent the future stratum vasculare.

This layer has been regarded by a few past authorities as remaining in the state of connective tissue cells at this stage, to change into muscle fibres only in fifth or sixth month embryo. But my ideal impregnated preparations show that they are already muscle cells, by the fact that they stain in the purple color peculiar to smooth muscle fibres and that they contain very minute muscle fibrils, though these spindle-form cells are far weaker in development than the typical

smooth muscle cells seen in the bladder and ureter. Outside this vascular layer is found a layer of similar spindle-form cells running longitudinally or obliquely, arranged very closely. This represents the future stratum supravasculare.

As described above, according to my observations, the tunica muscularis of the uterus in the fourth month embryo is already distinguishable into the three layers as in adult, the middle and outer layers already showing the nature of smooth muscle fibres.

The vagina in a fourth month embryo is already provided with an inner cavity all over, and connects with the uterus cavity proximally and with the vestibule through the vaginal orifice distally. The development of the vagina is very remarkable, the cross-section showing a higher development than the bladder, and the fold formation can be observed by naked eye. In the posterior wall of the vagina, papilla formation is conspicuous. The epithelium is composed of stratified flat epithelium with 20 to 30 rows, the outermost cells being nuclear and showing no cornification. The epithelium somewhat increases in thickness as the vaginal orifice is approached.

The tunica propria is represented by a close arrangement of connective tissue cells with an oval nucleus. No submucous layer to speak of is formed around it, but in the proximal part, especially in its posterior wall, a connective tissue layer of weak development containing venous plexus is found covering the tunica propria. The propria is formed very narrow where it comes into contact with the rectum in the rear. The tunica muscularis is formed by long cells with spindle-form nucleus, and runs in main circularly, but some longitudinal or oblique running is observable.

The epithelium decreases in thickness at once on running into the vaginal vestibule, thinning into a stratified flat epithelium of 10 rows of cells, which further decreases in the labium minus to 5-6 rows, and in the labium majus, dwindles down to an epidermis composed of epithelial cells of 3 rows or so with cornification. The ground substance of the labium minus consists of connective tissue containing spindle-formed smooth muscle fibres in places, and a large quantity of blood vessels, particularly, venous plexus, is found therein.

The clitoris is entirely covered with praeputium, the epithelium of the inner lamella thereof being common with the epithelium clitoridis. The epithelium of the outer lamella of the praeputium consists of a thin stratified flat epithelium of about 3 rows, showing cornification already. On the contrary, the inner common epithelium is of considerable thickness, with the surface facing the clitoris being generally smooth, but the outer surface is very rugged, due to the presence of round projections of varying size. The corpus cavernosum clitoridis is composed of closely arranged spindle-form connective tissue cells, containing few small blood vessels, and scarcely a venous plexus.

I herewith conclude my description of the histological image of the urogenital organs in fourth month female embryo and proceed to the statement of my observations on the innervation of these organs, limiting the details to the field of sensory nerve fibres.

The presence of sensory fibres in the ureter has been presumed clinically from the earlier days. Recently, Yokoyama[1] succeeded in proving histologically their presence in the distal part of ureter of human adult. But no statement on the subject by human embryos has been found hitherto. On the sensory innervation of the bladder, a study was conducted by Michailow,[2] using methylene bluem ethod by various animals, and recent ly Yokoyama[1] published a detailed report on the subject by human adults. Iwasaki[3] reports on his study on bladders of 7th month and

10th month embryos. These studies resulted in throwing good light on the subject.

According to my observation, close on the adventitia of the posterior wall of the bladder, especially outside the orifice of the ureter, a markedly developed nerve plexus containing numerous ganglia, the so-called plexus vesicalis is formed. This plexus is chiefly composed of sympathetic fine non-medullated fibres, but contains a few of thicker fibres too. The latter are to develop into medullated sensory fibres later on, but in a fourth month embryo, the presence of myelin is not to be clarified.

In the adventitia in the distal part of the ureter, a weak formation of nerve plexus originating in small nerve branches from the bladder plexus is found. This plexus sends out small branches into the muscularis, among which a small number of thick sensory fibres is seen. They run into the propria, and in most cases end closely beneath the epithelium bluntly or sharply without branching, but sometimes also in simple branched terminations, as shown in Fig. 1. In adult bladder, a small number of intraepithelial fibres are found too,—much smaller in number than in the bladder,—and in the propria, some glomerular terminations are found, though on rare occasions (Yokoyama).[1] But such terminations are not yet observed in my fourth month embryo.

The sensory innervation of the bladder in a fourth month embryo is as described in the following. Comparatively large nerve bundles originating in the plexus vesicalis run into the muscularis through the adventitia to form intramuscular plexus there, whence minute branches spread out into the submucosa. These branches often contain thick sensory fibres beside fine vegetative fibres. But the sensory fibres are very small in number, as Iwasaki[3] has also observed in 7th and 10th month embryos. They mostly end in the submucosa without branching, but rarely also branched terminations are found. Even a fewer number of them reach into the propria. I could find an extremely small number of them penetrating further into the epithelium as intraepithelial fibres (Fig. 2). Iwasaki[3] classified the sensory terminations into the three types of zigzag-formed, loop-like and recti-linear terminations in his 7th and 10th month embryos, but in my fourth month embryo I could not yet confirm such a classification.

The innervation of the adult male urethra has been clarified to a great extent by the studies of Seto[4] and Nobuta[5] recently, but no such study hat been made on female urethra. My observation on the urethra of a fourth month female embryo has been as follows.

The nerve bundles in the very weakly developed vegetative nerve plexus formed in the connective tissue around the urethra contain a small number of thick sensory fibres, mostly coming in form the outside of the urethra. These sensory fibres run into the propria together with the fine

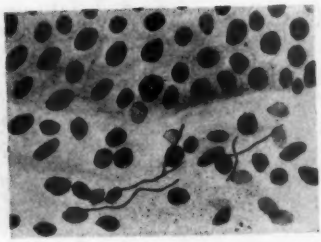

Fig. 1. Unbranched and simple branched sensory terminations found in tunica propria of distal part of ureter. 4th month female embryo. Seto's impregnation. ×800, reduced to 2/5.

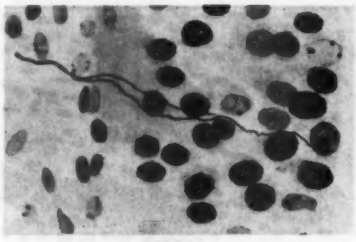

Fig. 2. Bifurcated intraepithelial fibre penetrating the epithelium in the base of bladder. Same embryo. Same staining. ×500, reduced to 1/2.

vegetative fibres or singly. How their terminations are represented I could not observe with clarity, but there is no doubt that the terminations are of unbranched or very simple branched type (Fig. 3). At this embryonic stage, of course, no terminal bodies, as the genital nerve bodies in adult urethra, and no intraepithelial fibres ending in the epithelium were observable.

It is an important question clinically whether sensory fibres are found in the uterus, but no one has proved anything about the subject. Only Herlitzer[6] in 1898 found existence of thick medullated fibres running out in comparatively complex branched endings beside the nerev fibres proper to the uterus, in his methylene-blue stained preparations, and thought these to be cerebrospinal in nature, but failed to assert that they were sensory. Dahl[7] (1916) also states that some medullted fibres are found among the nerve fibres in the uterus.

In my preparations, I found many nerve bundles running into the muscularis through the adventitia from the nerve plexus around the cervix uteri, which is connected with the plexus in the bladder. These bundles branch out into smaller bundles upon entering the muscularis and distribute themselves here and further in the propria. They contain a few thick fibres beside the fine vegetative fibres. The former penetrate into the connective tissue layer beneath the epithelium and end in sharp unbranched terminations, as shown in Fig. 4, or in extremely simple branched terminations. They must be sensory in nature and they are presumed to develop into well medullated nerve fibres, which were reported by Herlitzer[6] and Dahl.[7]

As seen above, I could ascertain the existence of nerve terminations, presumably of sensory nature, in the propria of the uterus in a fourth month embryo, though the number was very small indeed. However, as no study

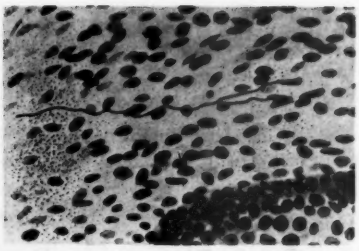

Fig. 3. Sensory nerve fibres running in tunica propria under the epithelium of urethra. Same embryo. Same staining. ×500, reduced to 1/2.

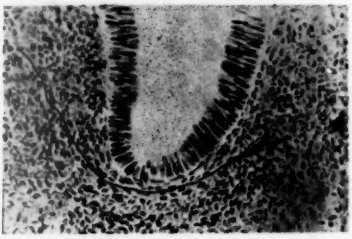

Fig. 4. An unbranched sensory termination found beneath the epithelium of uterus. v vegetative nerve fibres. Same embryo. Same staining. ×320, reduced to 2/5.

on the innervation of human adult uterus, utilizing the silver method used in this laboratory, has been reported, I have only to surmise that the adult uterus is also provided with such sensory terminations.

Recently, Ikui[8] of this laboratory reported his minute study on the innervation of adult vagina, vestibulum vaginae and labium minus, wherein he says that the sensory fibres in vagina are very sparsely distributed, only its distal part being provided with sensory terminations. This fact is in accordance with the extreme unsensitiveness of the mucous membrane of the vagina. But upon transition from the vagina proper to its vestibule, the development of the sensory fibres becomes markedly stronger, and more so in going over into the labium minus. In the latter may be found complex bush-like terminations and even genital nerve bodies, beside the simple branched terminations.

In my fourth month embryo, I found formation of vegetative nerve plexus in the adventitia of the vagina, which was more marked in its proximal part. The nerve plexus located in the lateral walls often contains a small number of sensory fibres. What is of special interest is the fact that these sensory fibres almost entirely run into the muscularis of the urethra adjacent to the front of the vagina, and never into the wall of the vagina. Thus the vaginal wall is almost devoid of sensory supply, but in the vicinity of the vaginal orifice, sensory fibres are found penetrating the muscularis and running into the propria, as Ikui[8] found it to be the case with adult. But their terminations are much simpler than those in adult, mainly of unbranched type as shown in Figs. 5 and 6, but rarely represented by simple branched terminations.

In the vestibulum vaginae, the sensory fibres are more abundant than at the lowest part of the vagina, ending in the propria mostly in unbranched terminations, as shown in Fig. 7. In the labium minus, the number of sensory fibres increases again, the connective tissue under the epithelium

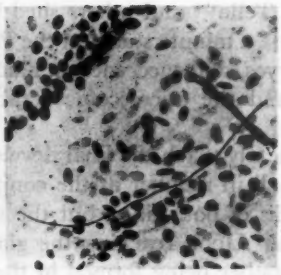

Fig. 5. An unbranched sensory termination found in tunica propria of vagina near the vaginal orifice. v vegetative nerve fibres. Same embryo. Same staining. ×500, reduced to 2/3.

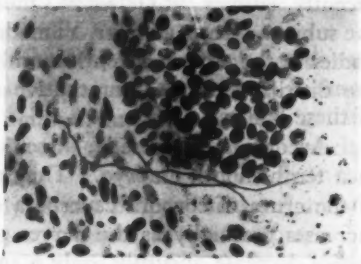

Fig. 6. Unbranched sensory terminations found in a papilla in the vaginal orifice. Same embryo. Same staining. ×500, reduced to 2/3.

of the inner lamella being particularly provided with many sensory fibres, which end in unbranched or simple branched terminations. But at this embryonic stage, no formation of complex branched terminations or genital nerve bodies, as found in adult genitals, is observable, neither any intraepithelial fibres.

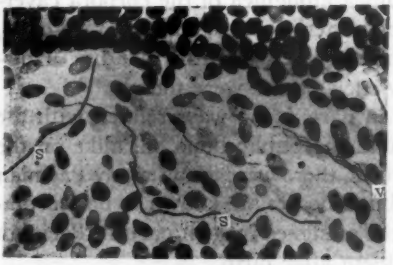

Fig. 7. Unbranched sensory terminations (s) and vegetative nerve fibres (v) found beneath the epithelium of vestibulum vaginae. Same embryo. Same staining. ×500, reduced to 1/2.

Recently Yamada[9] made a minute study on the innervation of clitoris, using tenth month embryos as material. I collated my observations on my fourth month embryos with his and succeeded in obtaining interesting results as follows.

The sensory fibres entering the clitoris all originate in n. dorsalis clitoridis. This dorsal nerve progresses along the dorsal and outer side of the tunica albuginea of the corpus cavernosum clitoridis together with its many branches, as is also the case with Yamada's[9] tenth month embryos, reaching the basis of glans clitoridis, a part thereof into the praeputium clitoridis and labium minus, yet another into the glans clitorides, especially into its propria beneath the epithelium. It was known from early times that Pacinian bodies are formed in the course of the dorsal nerves. More

recently, studies by Geller,[10] Ohmori,[11] Tello[12] and others shed light on the subject. In particular, Yamada[1] made a minute study on the Pacinian bodies in the clitoris of tenth month embryos, and succeeded in elucidating the distribution, the quantitative data and the microscopic construction of these bodies.

According to Yamada, these bodies nearly reach the final development in a tenth month embryo. They are small in number at the conjunction of the crura of corpora cavernosa clitoridis of both sides, but the number increases gradually as the nerves pass on. Upon reaching the glans, the bodies decrease abruptly, only a small number being formed in the glans, and a few being found dispersed in the praeputium. There are found, however, in the tenth month embryo, some Pacinian bodies still in the stages of imcomplete development. From this view-point, it may be said that the Pacinian bodies in genitals of tenth month embryo are in somewhat weaker development than in the palm of the hand in the same old embryo, which are said to be in quasi-complete stage of development, according to Wada.[13]

In my fourth month embryo, it is of interest that at this early stage, the Pacinian bodies are formed in many places. But in quantity, these are very smaller than in Yamada's tenth month embryo, being found only in very sporadic formation outside the tunica albuginea of the corpora cavernosa clitoridis, especially in their outer posterior parts. But in rare cases, the bodies were also found formed in the corpus cavernosum glandis. However, in the praeputium, scarcely any such bodies were found.

I could observe these bodies also in parts in no ways related with the clitoris. In the fascial formation around the public bone, all alike on the side facing the skin, the vagina and rectum, there were found many Pacinian bodies in formation. Seeing that Pacinian bodies are known to be receptacles responding to pressure sensation, the existence in such parts of Pacinian bodies presents a very interesting problem after birth.

The Pacinian bodies found around the corpora cavernosa clitoridis have only 2 or 3 lamellae, as shown in Fig. 8, the inner bulb being very young and small in width, and the nerve fibres running into it ending bluntly, without branching. The bodies found around the pubis are also very undeveloped. In short, the Pacinian bodies in the clitoris of the fourth month embryo are, though found in formation, only in a primary stage and small in number, to be developed gradually thereafter and to reach the nearly complete development in tenth month embryo, as observed by Yamada.[9]

As shown above, only Pacinian bodies are formed in the embryonic stage, but other corpuscular terminations are scarcely formed yet, as may be inferred from the accumulated results of related studies in this laboratory.

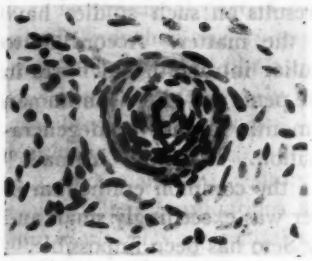

Fig. 8. Pacinian body found outside the tunica albuginea of corpus cavernosum clitoridis. Same embryo. Same staining. ×600, reduced to 1/2.

Accordingly, in my study, no trace of genital nerve bodies were found.

The sensory terminations beside the Pacinian bodies in the clitoris in a fourth month embryo are represented as unbranched or sometimes as simple branched terminations. They are formed most conspicuously in the propria in the glans and the connective tissue of preaputium. For example, as shown in Fig. 9, 2 or 3 thick sensory fibres run toward the propria through the corpus cavernosum glandis, finally to end without branching or after only one bifurcation. However, not rarely, in the proximal parts of n. dorsalis clitoris, very thick sensory fibres are found dividing into several branches, as shown in Fig. 10. This fact shows that one thick sensory fibre is capable of passing into many terminations—a very interesting phenomenon. It is thought that the branches of such particularly thick fibres take part in formation of strong genital nerve bodies later on.

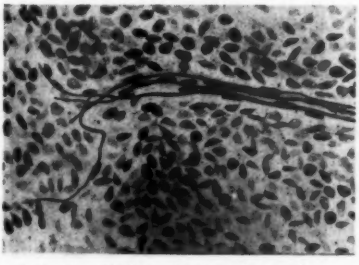

Fig. 9. Unbranched and simple branched terminations ending in the propria beneath the common epithelium of glans clitoridis. Same embryo. Same staining. ×400, reduced to 2/3.

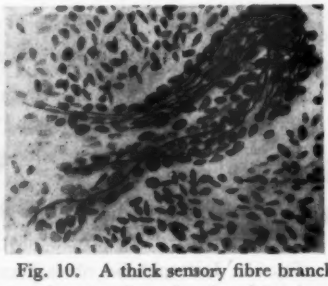

Fig. 10. A thick sensory fibre branching out in many branches found in corpus cavernosum clitoridis. Same embryo. Same staining. ×320, reduced to 2/3.

In conclusion, I will speak of the intraepithelial fibres existing in the epithelium common to the inner lamella of the praeputium and the galns clitoridis as a type of sensory terminations found in the clitoris. They were described by Retzius[14] in 1894 in his study of the clitoris of a 28 cm. human embryo, but as his preparations were stained with the old Golgi's method, the exactitude of his observation may be well doubted. More recently, Ohmori[11] and Tello[12] made reports thereon, but since the silver

method was perfected by Seto[4] (9139), the results on such studies have made powerful contributions in elucidating the matter. According to Seto, there are found two types of very peculiar intraepithelial fibres in the above mentioned common epithelium in the penis and clitoris in human embryos, which are best developed in the 7th month, to undergo degeneration later on and to disappear after parturition. Recently, Yamada[9] proved the existence of intraepithelial fibres in the common epithelium in clitoris of tenth month embryo, but the number was exceedingly small and the classification of them into the two types by Seto has been impossible.

In my study on the fourth month embryo, I thought that the intraepithelial fibres were not yet formed, seeing that the common epithelium itself was only very weakly developed, in comparison with the seventh month embryo, but to my astonishment, I found the intraepithelial fibres in unexpected growth. However, their number was very small and not at all comparable with that of the seventh month embryo. But what must be especially noted is the fact that the two types of terminations (Seto) are already clearly discernible at this stage of development. The first type (Fig. 11) consists of fine fibres, which often divide into 2 or 3 branches, and end in general sharply or bluntly after running irregular but peculiar winding courses. The second type (Fig. 12) is formed in the epithelial cell

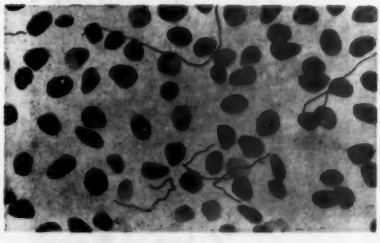

Fig. 11. Intraepithelial fibres Type I found in the common epithelium of glans clitoridis. Same embryo. Same staining. ×800, reduced to 1/2.

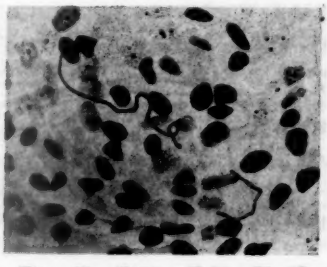

Fig. 12. Ditto, Type II. Same embryo. Same staining. ×800, reduced to 1/2.

gatherings arranged in centripetal rings. The nerve fibres are much more powerful than in the first type, show many variations in size and often give out a few branches, which end sharply after running irregular winding courses.

Summary

The innervation of urogenital organs in a fourth month female embryo may be outlined as follows.

In the distal part of the ureter, unbranched, and on rarer occasions, simple branched sensory terminations may be found in the propria. However, intraepithelial fibres and glomerular terminations are not yet formed.

In the bladder, only unbranched and simple branched terminations are observed in formation. In rare cases, intraepithelial fibres were proved to exist.

In the urethra, sensory fibres run into the propria from the outside wall. Their terminations are also unbranched and simple branched terminations, and no genital nerve bodies nor intraepithelial fibres were observed.

In the propria of the uterus, a small number of unbranched terminations are found. This fact seems to indicate the presence of sensory terminations in adult human uterus.

In the vaginal wall, scarcely any sensory fibres are verified. Only near the outer orifice, a small number of unbranched terminations were observed. In the vestibulum vaginae, the number of sensory fibres is much larger, the terminations being mostly of unbranched type. In the labium minus, the number increases further, unbranched and simple branched terminations being in evidence, but no complex branched terminations nor genital nerve bodies are found.

As sensory terminations in the clitoris, Pacinian bodies may be mentioned first. Their number is yet very small, and their formation is found around the tunica albuginea of the corpus cavernosum clitoridis, and on rare occasions, in the corpus cavernosum glandis. It is interesting to note that Pacinian bodies are formed in places in the fascial formation around the pubis, unrelated with the clitoris. These Pacinian bodies are naturally in the inchoate stage of development by fourth month embryo.

In the clitoris, no genital nerve bodies are found, not even a trace of them. Sensory terminations other than the Pacinian bodies are represented by unbranched and extremely simple branched terminations, which are formed in the tunica propria glandis and the connective tissue in the praeputium in particular. In the terminal parts of the branches of n. dorsalis clitoridis, frequently very thick sensory fibres are seen branching out in several branches, which seems to show the fact that one thick nerve fibre may run out in a number of nerve terminations, or probably in many genital nerve bodies.

In conclusion, I may mention the interesting fact that intraepithelial fibres are already seen in formation in the common epithelium between the inner lamella of the praeputium and the glans clitoridis, although, maybe, in a much lower stage of development than in a seventh month embryo (Seto), and that they are clearly distinguished into two separate types (Seto) in formation.

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Histological Study on Innervation of Pars mucosa of Vestibulum nasi in Human Embryo

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In 1944, Seto and Sada[1] succeeded in throwing light on the microscopic structure of adult human nasal vestibule, in particular, of its mucous membrane, and gave out a very interesting report on its innervation. Since then, I know no other report on the subject. I made up my mind to make a genetic study on the subject, and conducted a research on the nasal vestibule of human embryos, especially on its innervation.

The materials I took from 3 each of 6th and 10th month human embryos, which I fixed for more than half a year in 10% neutral formol, and prepared from them a series of 40 μ sagittal and frontal frozen sections, which I stained by means of Seto's silver impregnation. These numerous and beautifully stained preparations I subjected to a minute examination under a microscope and arrived at the observations stated in the following.

Experimental

1. The microscopic anatomy of vestibulum nasi

The fine structure of adult human nasal vestibule has been treated only by a few investigators in the past, and much has been left as yet unclarified. When Seto and Sada[1] of this laboratory made a study on the innervation of this part, they reported also on the microscopic structure thereof. I made a review of their numberous silver-impregnated pregparations of adult human nasal vestibule preserved in this laboratory in preparation to my study and could confirm their views through my own observation.

In outline, the wall of vestibulum nasi in human adult is divisible into the cutaneous part provided with hairs and the mucous part succeeding it to the inside, the latter again into the corneal, the non-corneal and the transitory parts, according to the various natures of the epithelium from the frontal area backwards. The relative areas of these various parts, however, are very unequal locally as well as individually. The formation of papillae from the tunica propria into the epithelium is very marked in the corneal part, but becomes much poorer in the rear.

In following up the above observations with the examination of the same mucous part in embryonic development in comparison, I found that the nasal vestibules in 6th and 10th month embryos showed an unexpectedly advanced development, so that in outward appearance, they showed little difference from adult specimens, and the division into the haired cutaneous part

and the mucous part succeeding it was fairly distinct, for the latter was unhaired, showed peculiarity in epithelium, and papillae and nasal glands were formed therein.

The mucous part of the vestibulum nasi in 6th month embryos is surfaced with stratified flat epithelium generally thicker than in the cutaneous part in the part adjacent thereto, corresponding to Seto and Sada's corneal part in adult. The surface of the epithelium showed corneal scale or lamina formation, and beneath the epithelium, the papillae were found in incipient formation, though incomparably more infantile in development than those in adult specimens. Thus it is beyond doubt that this part corresponds to the corneal part in adult man and is already nearly formed by the 6th month of embryonic life.

On the other hand, it is very interesting that in the 6th month embryos, the part corresponding to the non-corneal part in adult is not yet completed as described as follows. The mucous membrane in this part is surfaced with a rather thick stratified columnar epithelium in 10–12 cell layers, which is distinctly demarkated at the innermost limit from the low ciliated epithelium of 2–3 cell layers of the pars respiratoria of cavum nasi. That is, the epithelium of the respiratory-part, as is the corneal part, is completed as the ciliated epithelium as in adult, while the non corneal part alone remains in the high stratified columnar epithelium, before its development into the final stage of stratified flat epithelium, as is the case with the development of the epithelium of oesophagus. But as the basal surface is generally even, undented by the formation of papillae, it is doubtless that this part corresponds to the noncorneal part in adult.

The change of the stratified columnar epithelium into stratified flat epithelium would have to be accomplished by the metamorphosis of the columnar cells in the surface layer of the stratified columnar epithelium into a flat form. However, in a small area of the epithelium adjoining the inner respiratory part, it appears, the columnar form will persist throughout, to become the boundary or transitional part in the adult.

The vestibulum nasi in 10th month embryos is a little more advanced in development than in 6th month embryos, as described hereunder. However, the development is not yet in the finally perfected stage, as in the adult. Namely, in the mucous part of vestibulum nasi in 10th month embryos, the corneal part surfaced with corneal stratified epithelium is more developed, especially in distribution, than in a 6th month embryos, and the papillae beneath it are larger both in size and number.

The boundary between the corneal part and the non-corneal part succeeding the former is shown by the distinct transition of the corneal epithelium into the non-corneal flat epithelium. The formation of non-corneal flat epithelium in the non-corneal part, however, is observed only in the foremost half of the non-corneal part, though in some places the breadth shows some fluctuation, and the remaining rear half is surfaced, just as in 6th month embryos, with stratified columnar epithelium. Also the basal surface of the epithelium is smooth and even, and almost, no papillae are formed thereunder.

In short, the evolution in the mucous part of the nasal vestibule in the 10th month embryo, be it noted, has progressed to the stage of non-corneal stratified flat epithelium formation in the foremost half of the non-corneal part, but the hindermost half is as yet under columnar epithelium, so that this part is supposed to turn into flat form gradually after birth.

Now, the distributing ratio of the areas of the corneal and non-corneal parts show considerable inequality locally and individually, in embryos as well as in adults (Heiss[2]), Seto and Sada[1]), and these parts are usually arranged unsymmetric even in the right and left nasal vestibules of the same individual.

In the mucous membrane of the nasal vestibule in adult are found very well-developed nasal glands, compound glands consisting of a small number of mucous glands and a larger number of serous glands being found in the submucosa. Their ducts are also remarkably well-developed, often showing an ampullar swelling. The nasal glands are best developed in the

asal septum, where they are found even in the frontal part of the corneal part. On the other hand, the development of the nasal glands in 6th month embryos is so poor, that they are often not to be found even in the innermost part of the nasal vestibule. In 10th month embryos, however, they are so remarkably developed to be seen advanced at least to the central part of the non-corneal part. None is as yet seen more in front, certainly not in the corneal part, so that it must be concluded that the nasal glands here in the corneal part develop gradually after birth.

2. The innervation of the nasal vestibule

In the mucous membrane of the nasal vestibule, the plexus submucosus is formed already in the 6th month, and in more marked development in the 10th month of the embryonic existence. This is in good development in the non-corneal part, but is poorer in the corneal part. The nerve elements composing this plexus are in majority unmedullated vegetative fibres, the remainder consisting of medullated sensory fibres. The vegetative fibres in the minute nerve bundles sent out from the submucous plexus toward the propria come into anastomosis at many points with the vegetative fibres running along the small blood vessels, in particular, the small artery walls. The above observation is not much at variance with that on the nasal vestibule in adults, so that it is clear that the general arrangement of the nerve fibres here is almost perfected in the latter half of the embryonic life.

The termination of vegetative fibres here in the mucous membrane of the nasal vestibule is also represented by Stöhr's terminalreticulum completed already in the latter stage of the embryonic life, as many studies of this laboratory have established. The terminalreticulum spreads out for the nasal glands around their end parts and ducts and control these by contact. For the blood vessels, in particular, capillaries in the propria, the connective tissue and epithelium it stands in the same control modality. On nearer examination, the vegetative fibres are especially well -developed in the corneal part where papillae are formed into the epithelium, being less developed in the non-corneal part where papillae are not formed. That is to say, the development of vegetative terminalreticulum is in parallel with the formation of papillae. But the main cause of such special development seems to lie in the remarkably well-developed capillaries in the papillae, rahter than in the existence of the papillae themselves.

In the latter stage of the embryonic life, the sensory fibres in the mucous membrane of the nasal vestibule are not so well developed. They run from the submucosa into the propria, generally to end directly under the epithelium, and it may be noted that many of them penetrate into and end in the papillae. Thus, the development of sensory fibres, as clarified by many other studies in this laboratory, is abreast with the development of papillae, in the nasal vestibule too.

As papillae are found in formation in the corneal part, in particular in

its frontal part, already in 6th month embryos, so the terminal formation of sensory fibres is most conspicuous in this area. On the other hand, in the non-corneal part, where the formation of papillae is scarcely seen, if anything, even in the last month of the embryonic life, the sensory fibres are much worse developed. In the former part, the sensory fibres are of medium or large size and their terminations are already somewhat differentiated, while in the latter part, the fibres are in general minutely thin, with endings of very simple type in the majority.

Anyhow, the sensory terminations in the nasal vestibule, in the latter stage of embryonic life, stand much behind those in adult man in development, mainly being represented by simple unbranched endings. Usually, one mudellated fibre loses its myelin upon entering the ending area, and gradually tapering off, comes to end in the vicinity of or touching the basal layer of the epithelium. Nextly, there are seen simple branched terminations, which are few in 6th month embryos, but exist in a relatively large number in 10th month embryos. Usually, in these elements, one medullated fibre, upon losing its myelin, divides into 2–3 rami, which end sharply beneath the epithelium, as with the unbranched endings above. In both the above, the nerve fibres are fine in the non-corneal part (Figs. 1 and 2),

Fig. 1

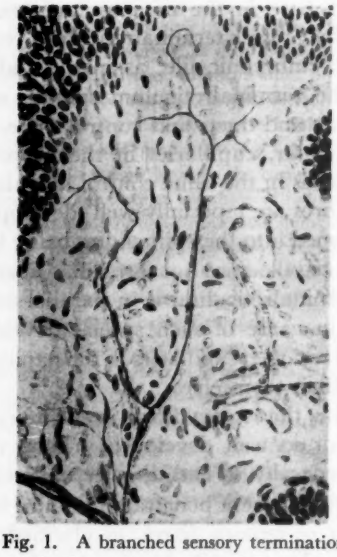

Fig. 2

Fig. 1. A branched sensory termination accompanying vegetative terminalreticulum, found in the tunica propria of the non-corneal part of mucous part of vestibulum nasi. 10th month human embryo. Seto's silver impregnation. ×300, reduced to 2/3.
Fig. 2. Ditto.

but very often medium-sized or thick in the corneal part, where the papillae are formed conspicuously (Fig. 3), particularly stout in the strongly developed papillae in the part nearer to the cutaneous part (Figs. 4 and 5).

Fig. 3

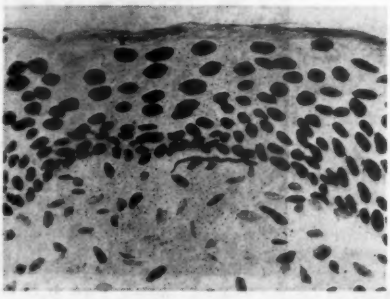

Fig. 4

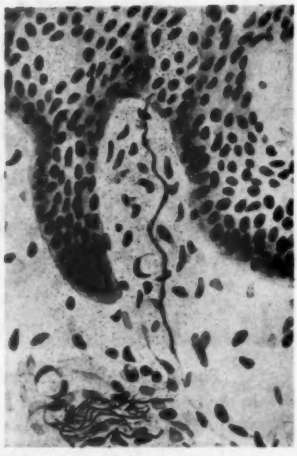

Fig. 3. A stout unbranched sensory termination found directly beneath the epithelium in the central area of corneal part of muocous part of vestibulum nasi. 10th month human embryo. Same staining. ×400, reduced to 2/3.

Fig.4. A thick unbranched sensory termination formed in a papilla in the frontal area of corneal part of mucous part of vestibulum nasi. 10th month embryo. Same staining. ×450, reduced to 2/3.

As the third type, in human adult, there were found the glomerular terminations, especially, the terminal bodies resembling the genital nevre bodies in the mucous part of the nasal vestibule, above all in the papillae of the corneal part (Seto and Sada).[1] However, neither in my 6th month nor 10th month embryos, I could find any such terminations in the same place, which fact is in conformance with the nonexistence of such terminal bodies in any corporeal part in human embryos established by many researches in this laboratory. But rarely, sensory terminations showing trap-like or simple glomerular arrangement of terminal fibres (Fig. 6). with relatively complex branching, which might be taken to develop into corpuscular endings later on, have been found in the well developed papillae in the corneal part in a 10th month embryo.

The fourth type of sensory terminations in the mucous part of the nasal vestibule is represented by the intraepithelial nerve fibres. In 1928, Kadanoff[3] first observed one such fibre, finer than any similar fibre found

Fig. 5

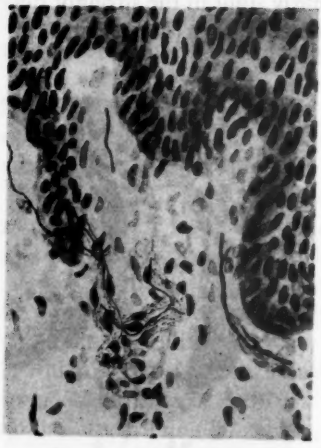

Fig. 6

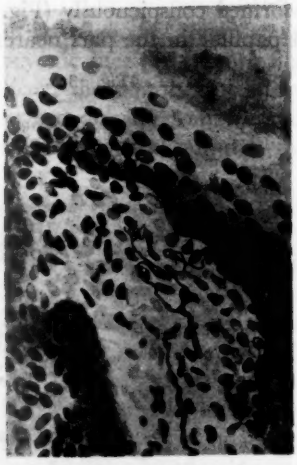

Fig. 5. A thick branched termination (in the right) formed in a papilla in the frontal area of corneal part of the same. The tips of the branches cut away. In the left, there are seen sensory fibres accompanying vegetative terminalreticulum. Same staining. ×450, reduced to 2/3.

Fig. 6. A simple glomerular termination found in a papilla in the frontal area of corneal part of the same. Same staining. ×400, reduced to 2/3.

in other parts of the body, in the outer nasal wall in human adult. However, upon examining his illustration of the fibre, I take it that it was found in the hindermost transitional part of the nasal vestibule, seeing that the epithelium in which it is embedded was stratified columnar. In the more recent and minute research by Seto and Sada,[1] it was found that such a minute fibre is seen running from the top of the papilla into the epithelium, in the case when the papillae are formed in the transitional part, while far more numerous intraepithelial fibres, a little different in nature, are found in the frontal non-corneal part and the corneal part. The intraepithelial fibres in the non-corneal part are thicker than those in the transitional part, often branching out into rami which end sharply or in a knob respectively. In the frontal corneal part, where the epithelium is covered with corneal scales, there are, beside the above type of intraepithelial fibres, some intraepithelial fibres of peculiar type, which is represented by stout but short fibres runnig into the extremely thinned epithelium formed by the intrusion of stout papillae, and ending bluntly or in a knob. There are also intraepithelial fibres in the ducts of the nasal glands, but these are all very simple in conformation and small in number.

As described above, intraepithelial fibres of various types are found in

all parts of pars mucosa of vestibulum nasi in adult human, so I have scrutinized my embryonic preparations, to find out if these fibres are found developed also in the latter stage of embryonic life. The results were as follows. Into the epithelium of the mucous part, sensory fibres are found to penetrate in the latter stage of the embryonic life already, but as they are very infantile in development and small in number, it must be assumed that the full-fledged development of intraepithelial fibres takes palce gradually after birth.

In 6th month embryos, the intraepithelial fibres are very few in number, are all minute in size and end unbranched after an only short course, while in 10th month embryos, they become larger than the former in number as well as in scale, but they are very thin in the non-corneal part, sometimes bifurcating, but in most cases ending unbranched (Figs. 7 and 8). As in this part, besides, many minute fibres are found ending in or

Fig. 7

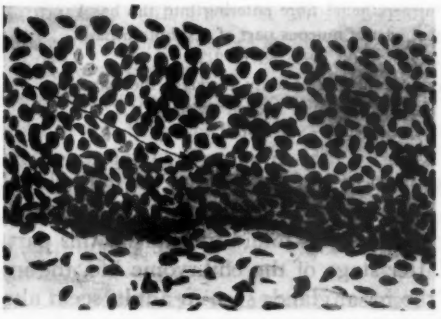

Fig. 8

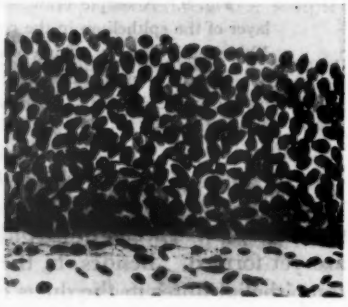

Fig. 7. A minute unbranched intraepithelial fibre. Non-corneal part of mucous part of vestibulum nasi. 10th month human embryo. Same staining. ×400, reduced to 2/3.

Fig. 8. Unbranched intraepithelial fibre ending in a small knob. Non-corneal part of the same. Same staining. ×400, reduced to 2/3.

touching the basal layer of the epithelium, it is probably supposed that they run further into the upper layer of the epithelium after birth. The intraepithelial fibres in the corneal part are more numerous in appearance, are generally thicker and end in simple, but in rarer cases, in somewhat more complex, branched endings (Fig. 9). Here also nerve fibres are here and there found to end in (Fig. 10) or touching the basal layer of the epithelium, which probably grow into intraepithelial fibres after birth.

As mentioned above, well-developed papillae are found in the frontal corneal part, and in these papillae, in most cases, very stout sensory fibres are observed running up to the tip, to end there sharply or bluntly (Fig. 4).

Fig. 9

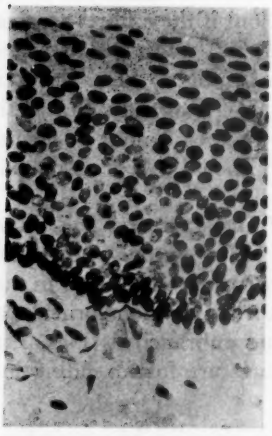

Fig. 10

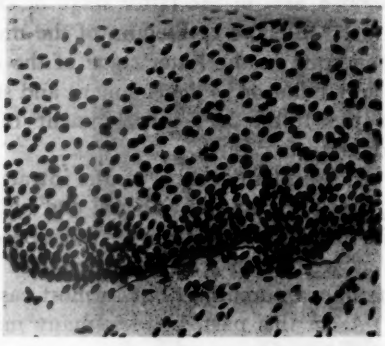

Fig. 9. A simple branched intraepithelial fibre entering into the basal layer of the epithelium in the corneal part of mucous part of vestibulum nasi. 10th month embryo. Same staining. ×500, reduced to 2/3.

Fig. 10. Intraepithelial fibres running horizontally through the basal layer of the epithelium. Corneal part of the same. Same staining. ×280, reduced to 2/3.

However, almost none of them are found to run further into the epithelium, to end as intraepithelial fibres. That is, in the embryonic life, no such stout and short special intraepithelial fibres, as found in adult in this part, are yet formed. Besides, in the latter stage of the embryonic life, the intraepithelial fibres in the ducts of the nasal glands seen in adult, seem also as yet unformed.

Summary

The nasal vestibule is already in good development in the latter stage of the embryonic life, and is divisible, as in adult, primarily into the cutaneous part overgrown with hairs and the mucous part succeeding it on the inner side. In the latter part, however, the distinction of the three parts of the corneal, the noncorneal and the transitional, distinguished by the nature of the epithelium and the formation of papillae, is not yet sufficiently clear. In 6th month embryos, the corneal part is nearly complete, but the non-corneal and transitional parts are both surfaced with thick stratified columnar epithelium, so that their boundary is not clear-cut. In 10th month embryos, the surface of the epithelium in the frontal half of the non-corneal part has changed to stratified flat epithelium, but the rear half remains as yet surfaced with stratified columnar epithelium, and the

boundary between the non-corneal and the transitional part remains yet undefined. Papillae are formed in considerable development in the corneal part, but almost none in the non-corneal part.

The end apparatus of the vegetative nerve fibres is completed as Stöhr's terminal-reticulum, and stands in tactile control against the nasal glands, the blood vessels, in particular, the capillaries.

There are comparatively few sensory nerve fibres supplying the mucous membrane of the nasal vestibule. Most of the fibres run into the lamina propria and end beneath the epithelium and some run further into the epithelium to end as intraepithelialfibres. As sensory fibres penetrate preferentially into papillae, the corneal part having papillae is provided with better developed sensory fibres than the non-corneal part without papillae. That is, the sensory fibres in the corneal part are generally thick and end in somewhat differentiated terminations, while those in the non-corneal part are mostly minute and end in simple ones. But these sensory terminations are as yet in much lower stage of development in comparison with those in adult, mostly being represented by the simple unbranched endings, especially in 6th month embryos.

The second type of sensory terminations is represented by the simple branched terminations composed of 2–3 rami which are found rather often in the 10th month embryos. The third type of sensory nerve endings is corpuscular in form, but such terminal bodies are as yet not formed in the latter stage of embryonic life. But the terminations showing a very simple glomerular arrangement of sensory fibres, presumably precursors of such bodies, are in some cases found in the major papillae in the corneal part.

The fourth type of sensory endings, the intraepithelial fibres, has been proved to exist in the latter embryonic stage, but their infantile form and small number seem to indicate their full-fledged development to be postponed till after birth. Namely, in 6th month embryo, the intraepithelial fibres are very few in number, are minutely thin and end unbranched after running a short course. In 10th month embryos, these fibres appear with greater incidence, and their terminal mode becomes more complex. For example, in the non-corneal part, there are seen often also bifurcated terminations, and in the corneal part, also branched intraepithelial fibres of medium size. Besides, in both the above parts, many sensory terminal fibres are found touching the basal layer of the epithelium or slightly penetrating it. These fibres probably develop into intraepithelial fibres in post-natal life.

Into the strongly developed papillae in the frontal area of the corneal part penetrate stout sensory never fibres to end there sharply or bluntly, but unlike those in adult, none of them has ever been found to run further

up into the epithelium, to end as the peculiar thick and short intra-epithelial fibre.

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Innervation of Pars cutanea of Vestibulum nasi and Nasus externus in Latter Half of Human Embryonic Life

By

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(Received for publication, January 10, 1954)

Since 1942, a series of histological studies on the innervation of the skin have been undertaken in this laboratory, which contributed to clarifying the interesting facts that in the haired skin, unlike the hairless skin, such as the palm and the sole, sensory nerve fibres are mostly developed in close relation with hairs, generally ending in complex terminations, while only a small minority of the fibres goes on close to the epidermis and end there in very simple terminations, and that the development of the sensory hair nerve fibres depends only on the locations, not standing in relation to the growth of the hairs.

The objectives of such studies on haired skin in this laboratory have been the scalp (Seto),[1] the eyelid (Seto).[2] the cutaneous part of lip in adult (Seto, Fujii and Ikui)[3] as well as in the latter half of embryonic age (Suga)[4] and the ventral abdominal wall in the 10th month human embryos (Azuma).[5] In succession to these, I had the opportunity of conducting the following study on the haired cutaneous part of the human nasal vestibule and the skin of the apex et ala nasi adjacent thereto.

The same preparations used in my study on the innervation of the mucous part of the nasal vestibule reported previously were reused in this study. These consisted of many sagittal and frontal sections of the nasal vestibules and tips of noses in 6th and 10th month embryos, stained with Seto's silver impregnation.

The sensory nerve fibres supplying the haired skin mostly terminate in connection with hairs as mentioned above. Their terminal areas are represented by specific locations, that is, Seto's so-called hair-nerve shields and tubes, consisting of special connective tissue cells. The former are formed one-sidedly in the follicle neck, while the latter many-sidedly, sorrounding the outer hair root sheath tubulously. Consequently, the development of the sensory terminations is stronger in the latter than in

the former. And as in the hair-nerve tubes the many-sided development of the sebaceous glands is prerequisite for the development of the sensory fibres, the latter is dependent very closely on the former. Needless to say, the development of the sensory fibres is also dependent on the density of the hair growth.

Since the short and stiff vibrissae in the cutaneous part of the nasal vestibule are rather sparse in growth, it would suffice with a comparatively small number of sensory nerve fibres. However, the sebaceous glands belonging to each vibrissa are always plural in number, which open into the hair root sheath through each duct, showing a many-sided strong development around the hair root sheath. It follows that the sensory terminal areas connected with the vibrissae are almost all represented by hair-nerve tubes. Consequently, the development of sensory nerve fibres must be strong too.

The above picture of tissue formation is clearly developed in the 6th month embryos already, becoming clearer in the 10th month, where the hair-nerve tubes consisting of spindle-shaped special connective tissue cells and the numerous sensory fibres running therein are very remarkably developed, as shown in Fig. 1. The terminal formations are illustrated in the vertical sections and are in general very complex, the majority being represented by plexus-like terminations, as shown in Fig. 2, and the minority

Fig. 1

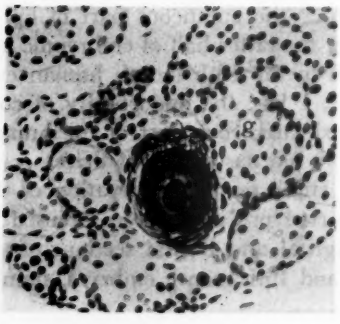

Fig. 2

Fig. 1. A number of sensory nerve fibres running through the hair-nerve tube formed around a vibrissa in the cutaneous part of nasal vestibule of a 10th month human embryo. Horizontal section. g sebaceous glands. Seto's silver impregnation. ×400, reduced to 1/2.

Fig. 2. Plexus-like termination of sensory hair nerve fibres formed in the special terminal area around a vibrissa in the same. Vertical section. Same staining. ×450, reduced to 1/2.

by serrate terminations (Fig. 3) or by a hybrid type of the two above, and sometimes by arborized terminations (Fig. 4). Not rarely, a part of the nerve fibres run upward from these terminations beyond the top of the follicle neck along the outer hair root sheath into the papillar layer, to end in very simple unbranched endings.

Fig. 3

Fig. 4

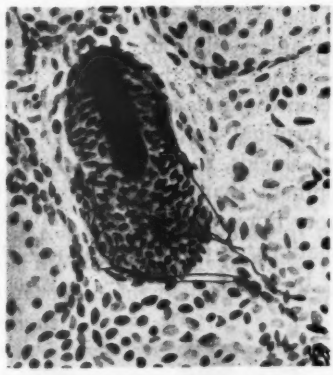

Fig. 3. Serrate termination of the same above. Same staining. ×900, reduced to 1/2.

Fig. 4. Arborized termination of the same above. Same staining. ×320, reduced to 2/3.

Seeing that the majority of the sensory terminations connected with the vibrissae in the cutaneous part of the nasal vestibule is formed complexly in the hair-nerve tubes, already in the latter half of the embryonic life, it may be presumed that they come to even further development in adult, to very complex terminal formations as may be found in connection with the eye-lashes (Seto)[2]

The epidermis of the cutaneous part of the nasal vestibule is of deciduous nature as in common skin and the papillar layer shows little tendency to form papillae into the epidermis. In consequence, there are very few sensory nerve fibres running into this part, only a few unbranched, sharply ending fibres being found beneath the epidermis in the papillar layer in rare cases.

The external circumferential part of the cutaneous part of the nasal vestibule, that is the margin of the nares, is visibly covered with dense, short downs, unlike the cutaneous part of vestibulum nasi. It follows that this area should be very rich in sensory nerve fibres, but as the sebaceous glands belonging to these downs are very weak and one-sided in development, not alike those to the vibrissae, some even almost unprogressed in

development at all, the hair nerve fibres distributed to this part end in very ill-developed hair-nerve shields, so that the sensory hair-nerve fibres are also small in number and terminate very simply, in most cases in simple plexus-like terminations.

In the apex and ala nasis, tow types of downs are found, the thicker and the thinner. The thicker downs grown in approximately regular distance, and the sebaceous glands belonging to them are strongly developed, with the exit of their ducts opening to the epidermis so large that they form pores quite visible to the naked eye, in adult. So, these downs show the appearence as if they beolng to the glands. However, as these strongly developed glands are formed only on one side of the outer hair root sheath, the terminal areas of the sensory nerve fibres are represented by hair-nerve shields, so that the sensory fibres themselves are far weaker in development than those belonging the vibrissae. The terminations of the sensory fibres are generally represented by simple plexus-like or arborized formations, as shown in Figs. 5 and 6, but sometimes serrate terminations may be found.

Fig. 5

Fig. 6

Fig. 5. Plexus-like termination in a hair-nerve shield around a minute down on ala nasi of a 6th month embryo. Same staining. ×600, reduced to 1/2.

Fig. 6. Arborized termination composed of thick nerve fibres formed in a hair-nerve shield of a minute down on ala nasi of a 10th month embryo. Same staining. ×450, reduced to 1/2.

The sebaceous glands belonging to the shorter downs are very poorly developed, as in the margin of the nares, some barely formed on the one side, or in rarer cases on many sides, but many of them showing no develop-

ment at all. In parallel, the sensory fibres and their terminations are very infantile, the majority having no end formation to speak of. Presumably, they are destined to show little further development even in adult stage, as the terminations of hair-nerve fibres to the minute downs in the cutaneous part of the eye-lid (Seto).[2)]

As few papillae are formed under the epidermis on the apex and ala nasi, the sensory nerve fibres ending in these parts are also very small in number and only unbranched terminations are found. But in some cases, a part of the hair-nerve fibres rises along the outer hair root sheath beyond their terminal area into the papillar layer to end there in simple sharp endings.

In summary, it may be noted that the cutaneous part of the vestibulum nasi, where the hair growth is only sparse, is not richly provided with sensory nerve fibres, but as in the majority of the hair follicle necks, there are found Seto's hair-nerve tubes originating in the development of the sebaceous glands formed on many sides, the development of sensory hair nerve fibres is good on this score, their terminations being all represented by complex plexus-like or serrate formations.

In the margin of the nares, which is densely overgrown with minute downs, the sensory nerve fibres should be abundantly found, but as the development of sebaceous glands is poor, the development of the hair-nerve terminations is much poorer than that in the vibrissae.

On the apex and ala nasi, two kinds of downs are found the thicker and the thinner. For the former, the sebaceous glands are strongly, but only one-sidedly developed, so that the sensory nerve fibres thereto end in rather simple plexus-like endings. As the sebaceous glands to the thinner downs are similar to those in the marginal part of the nares in their weak development, the development of sensory nerve fibres to these downs is also very poor.

Since few papillae are formed under the epidermis in the cutaneous part of the vestibulum nasi and the skin of the nose, as in the common haired skin, the papillar layer in these parts is also very poorly provided with sensory fibres, simple unbranched sharp terminations only being found on rare occasions.

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A Method of Colorimetric Estimation of Noradrenaline and Adrenaline in a Mixture and its Application to the Medulli-Adrenal Extracts

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(Received for publication, January 15, 1954)

The presence of noradrenaline in the adrenal gland together with adrenaline is nowadays well established. In fact, Bergström, Euler & Hamberg[1] were able to isolate L-noradrenaline as a crystalline base from the bovine adrenal gland.

For the estimation of noradrenaline in the adrenals some biologic methods were employed. These methods depend on the quantitative differences in the activities of adrenaline and noradrenaline on certain biologic preparations such as the isolated uterus of the non-pregnant rat, the cat blood pressure and so on. Although the biologic methods are superior to the chemical methods in the sensitivity, they cannot give precise values for the errors inherent to the methods. Accordingly, the methods of colorimetrical assay were also used by some investigators (Euler & Hamberg,[2] Auerbach & Angell,[3] Schuler & Heinrich[4] and others).

Among the chemical methods for estimating noradrenaline, the iodine method of Euler & Hamberg has been frequently used. Their method depends on a definite difference of oxidation rates between adrenaline and noradrenaline in oxidizing with iodine at pH 4. However, the method has practically a shortcoming that the colour tint of extracts after oxidation is not always similar with that of the standard solution, as already described by Euler & Hamberg themselves.

We[5] have already reported, as a preliminary note, an improved method to assay separately noradrenaline and adrenaline in a mixture by employing the permanganate reagent of Zanfrognini.[6] Using our method the colour tints after oxidation are almost identical.

It is the purpose of this paper to describe the details of the improved method and to report the experimental results concerning the noradrenaline content of the medullary tissues of the cattle and horse adrenals estimated by our method.

Experimental

(I)

Reagents

(1) Permanganate reagent: Made by dissolving 3 g. of potassium permanganate in 24 cc. of distilled water and adding 8 cc. of lactic acid solution (75%). The reagent so prepared is preserved in a dark cold place and renewed every five days.

(2) Hydrogen peroxide solution: 0.05% solution is made by diluting 3% hydrogen peroxide (Sankyo Co.) in water and renewed every day.

(3) Buffer solutions of pH 5.6 and pH 3.6: Made by mixing n solutions of sodium acetate and acetic acid.

(4) Adrenaline and noradrenaline solution: Made by dissolving 100 mg. of synthetic L-adrenaline or DL-noradrenaline base (Sankyo Co.) in 1/10 n HCl to make 100 cc. These solutions are kept in a refrigerator. Synthetic L-noradrenaline-D-bitartrate-monohydrate (Sterling Winthrop) dissolved with n/10 HCl in 1 : 1,000 is also used.

Method of Colour Development

0.1 cc. of the permanganate reagent is added to the sample to be tested. After some minutes 0.05% hydrogen peroxide solution is added in an amount just enough to remove the colour due to the permanganate reagent itself. This mixture is then diluted with cold water up to 6–10 cc. and the colorimetric determination is performed against the standard solution.

Effect of Different pH on Colour Development

In order to know the effects of different pH of solutions on the colour development of noradrenaline or adrenaline after adding the permanganate reagent the following experiments were made. To the solutions containing 100 μg. L-adrenaline or 100 μg. DL-noradrenaline, was added n-acetate-acetic acid buffer solution of pH 3.6 or pH 5.6. Permanganate reagent was then added.

After 1, 2, 3 or 5 minutes, the hydrogen peroxide solution was added. Diluting with cold water, the colorimetric determinations were then made with Duboscq colórimeter against the standard prepared by oxidizing 100 μg. adrenaline for 2 minutes. In all cases, the colour reaction was carried out at a constant temperature of 20°C. to avoid the influence of variation of the reaction temperature.

It was observed that an excess of hydrogen peroxide added has a slight bleaching effect on the fully developed colour and a slight intensifying effect on the colour of noradrenaline oxidizied at pH 3.6. However, these effects were fairly reduced by using hydrogen peroxide solution in an amount equivalent to 0.1 cc. of the permanganate reagent and by diluting

with cold water after oxidation.

The results obtained are given in Table I, where the figures show the colour density, taking the standard as 100.

TABLE I

Colour Development and pH

pH / Oxidation time (min.)		1	2	3	5
L-Adrenaline	pH 3.6	91	100	100	100
L-Adrenaline	pH 5.6	100	100	100	97
DL-Noradrenaline	pH 3.6		7	9	15
DL-Noradrenaline	pH 5.6		80	82	82

As shown in the table the colour density of noradrenaline oxidized at pH 5.6 attained its maximum, i.e. 82% of the standard within 3 minutes. At pH 3.6, adrenaline was fully oxidized within 2 minutes, whereas in the case of noradrenaline only 7% of the standard colour, that is 8.5% of the maximum colour of noradrenaline, was yielded during the same oxidation time.

In the experiments above mentioned, the colour density of noradrenaline oxidized at pH 3.6 was too small to be estimated colorimetrically. Accordingly, colorimetric comparisons were performed by adding some amounts of adrenaline to yield the colour enough for the estimation. Therefore, the colour density of noradrenaline oxidized at pH 3.6 were estimated by subtracting the colour density due to adrenaline added from that of adrenaline-noradrenaline mixture.

In the case of adrenaline no difference of colour tint was observed between those obtained at pH 5.6 and pH 3.6. The colour tint of noradrenaline oxidized at pH 5.6 was almost the same as that of adrenaline. However, the colour tint of noradrenaline oxidized at pH 3.6 was found to have slightly more brownish tone than that obtained at pH 5.6. To know the quality of the colour tint of brownish red yielded by oxidizing adrenaline or noradrenaline with the permanganate reagent, the absorption spectra of each colour yielded at pH 3.6 and 5.6 were determined using the electric spectrophotometer. It was substantiated by these determinations that the absorption spectrum of adrenaline oxidized at pH 3.6 as well as at pH 5.6 was qualitatively approximately similar with that of noradrenaline oxidized at pH 5.6. It was also noticed that the absorption curve of noradrenaline oxidized at pH 3.6 was somewhat different from that of adrenaline.

Colour Development and Reaction Temperature

The same procedure as described above was applied. The oxidation of 100 μg. adrenaline or noradrenaline with the permanganate reagent was performed at constant temperatures of 16°, 20° and 24°C. during various oxidation times. The standard was produced by oxidizing 100 μg. of adrenaline at pH 5.6. The data are listed in Table II.

TABLE II

Colour Development and Reaction Temperature

1) L-Adrenaline pH 3.6

Reaction temperature (°C.) \ Oxidation time (min.)	1	2	3	5
16	91	100		
20	91	100		
24	100			

2) L-Adrenaline pH 5.6

Reaction temperature (°C.) \ Oxidation time (min.)	1	2	3	5
16	100	100	100	100
20	100	100	100	97
24	100	100	100	96

3) DL-Noradrenaline pH 3.6

Reaction temperature (°C.) \ Oxidation time (min.)	1	2	3	5
16		5		7
20		7	9	15
24		13		20

4) DL-Noradrenaline pH 5.6

Reaction temperature (°C.) \ Oxidation time (min.)	1	2	3	5
16			77	
20		80	82	82
24		82	82	

The effects of reaction temperatures were hardly observed in the case of adrenaline oxidized during 2 minutes or more at pH 3.6 as well as at pH 5.6, whereas in the case of noradrenaline definite effects on the colour development were observed. These were found to be somewhat more marked at pH 3.6 than at pH 5.6.

Calibration Curves

To know the relation between the amount of adrenaline or noradrenaline and the colour density, the following experiments were carried out.

The solution of adrenaline or noradrenaline containing in amounts varying between 20 μg. and 200 μg. was adjusted to pH 3.6 or 5.6 and oxidized by adding the permanganate reagent. After 3 minutes at pH 5.6 or 2 minutes at pH 3.6, the hydrogen peroxide solution was added. Diluting the mixture with cold water, the colorimetic comparisons were made.

From these experiments it was elucidated that the calibration curves obtained by plotting the colour density against the amount of adrenaline or noradrenaline were approximately linear for both substances in amounts varying between 20 μg. and 200 μg.

Method of Differential Estimation of Noradrenaline and Adrenaline in a Mixture

As shown in Table I adrenaline is oxidized at pH 3.6 completely after 2 minutes, whereas only less than 10% of noradrenaline is oxidized during this time. At pH 5.6, however, noradrenaline as well as adrenaline can be oxidized fully within 3 minutes. Consequently, by means of the following procedure the amounts of noradrenaline and adrenaline in a mixture can be assayed separately.

0.1–1 cc. of a sample to be estimated or a standard adrenaline solution is measured. After being pre-adjusted, if necessary, to the neighbourhood of pH 3.6 or pH 5.6, respectively, it is adjusted to pH 3.6 or pH 5.6 by adding n-acetate-acetic acid buffer solutions. 0.1 cc. of permanganate reagent is then added. After 2 minutes (at pH 3.6) or 3 minutes (at pH 5.6), 0.05% hydrogen peroxide solution in an amount equivalent to the permanganate is added and then the mixture is diluted with cold water up to the volume of 6–10 cc. The oxidation with the permanganate is always made at a constant temperature of 20°C. to avoid the effects of a reaction temperature on the colour development. A comparison of the colour density is performed against the standard within 5 minutes.

The amount of noradrenaline and adrenaline can be calculated from the following formulas:—

$$A = X + n_{3.6} \cdot Y \qquad B = X + n_{5.6} \cdot Y$$

where X and Y are the amount of adrenaline and noradrenaline, respectively; A and B are μg. equivalents of adrenaline estimated at pH 3.6 and pH 5.6, respectively; $n_{3.6}$ and $n_{5.6}$ are the colorimetric activity ratio of noradrenaline to adrenaline estimated at pH 3.6 and pH 5.6, respectively.

The validity of this method was confirmed by the following experiments. The mixtures containing various amounts of adrenaline and noradrenaline were prepared and used as the test solutions. The noradrenaline and adrenaline contents of these test solutions were estimated with the above mentioned method. In these estimations it was noticed that the colour tint due to adrenaline used as the standard and mixtures of noradrenaline and adrenaline was approximately identical. The tabulation below shows an example of the results.

TABLE III

Adrenaline-noradrenaline mixture containing μg.		Chemical estimates μg.	
Adrenaline	Noradrenaline	Adrenaline	Noradrenaline
80	20	80.9	19.6
60	40	61.3	38.4
40	60	40.3	61.4
20	80	22.8	77.1

(II)

Using the permanganate method mentioned above, I was able to assay noradrenaline and adrenaline contents in the medullary tissue of the adrenals of cattle and horses. The adrenal gland was taken out after the slaughter at the abattoir as soon as possible. The medullary tissue was dissected from the cortex and was extracted with 2–4% trichloracetic acid. The noradrenaline and adrenaline contents were then estimated. In the estimation it was noticed that the colour tint of the extract after oxidation with the permanganate reagent was approximately similar with that of the standard adrenaline solution.

Bovine adrenals:

The medullary extracts were prepared from 32 adrenal glands. The estimates are shown in Table IV.

1.02–4.57 mg. noradrenaline and 4.57–9.47 mg. adrenaline per g. of the medullary tissue were found to be contained. The mean values were 2.89 mg. and 7.03 mg. per g., respectively. Noradrenaline was calculated to be contained in an amount of 29% (14–39%) of the total.

Horse adrenals:

With 4 medullary extracts of horse adrenal glands, noradrenaline and

TABLE IV

Noradrenaline and Adrenaline Content in the Bovine Adrenal Medullary Tissue, estimated by the Permanganate Method

No. of exper.	No. of animals Body weight & sex	Side of gland	mg. per g. of medullary tissue		Per cent noradrenaline
			Noradrenaline	Adrenaline	
1	1	r	2.19	6.38	26
2	390 kg. ♂	l	1.62	7.28	18
3	2	r	2.93	6.61	31
	350 kg. ♀				
4	3	r	3.44	6.71	34
5	390 kg. ♀	l	2.52	5.33	32
6	4	r	2.42	7.20	25
7	480 kg. ♂	l	2.20	6.68	25
8	5	r	3.29	7.93	29
9	490 kg. ♂	l	3.60	5.89	38
10	6	r	1.40	7.22	16
11	490 kg. ♂	l	1.02	6.28	14
12	7	r	3.74	8.03	32
13	430 kg. ♂	l	1.70	7.79	18
14	8	r	4.57	6.98	39
15	400 kg. ♀	l	3.11	5.95	34
16	9	r	3.52	7.88	31
17	400 kg. ♂	l	4.33	7.40	37
18	10	r	3.34	8.08	29
19	400 kg. ♂	l	2.99	9.47	24
20	11	r	2.59	6.65	28
21	470 kg. ♂	l	3.01	4.96	38
22	12	r	3.13	6.40	33
23	470 kg. ♂	l	3.24	5.38	38
24	13	r	3.39	7.59	31
25	400 kg. ♀	l	2.43	8.57	22
26	14	r	2.49	7.92	24
	360 kg. ♂				
27	15	r	2.33	7.22	24
28	480 kg. ♂	l	2.71	4.57	37
29	16	r	3.64	8.55	30
30	460 kg. ♂	l	3.45	8.46	29
31	17	r	3.03	7.58	29
32	410 kg. ♂	l	3.21	6.18	34
Max.			4.57	9.47	39
Min.			1.02	4.57	14
Mean			**2.89**	**7.03**	**29**

adrenaline contents were estimated. The results are displayed in Table V. The values obtained were 1.47–2.63 mg. noradrenaline and 7.35–10.47 mg. adrenaline per g. of medullary tissue, the average values being 1.95 mg. and 8.70 mg., respectively. The relative amount of noradrenaline was calculated to be 18.5% (12–26%) of the total.

TABLE V

Noradrenaline and Adrenaline Content in the Horse Adrenal Medullary Tissue, estimated by the Permanganate Method

No. of exper.	No. of animals body weight & sex	Side of gland	mg. per g. of medullary tissue		Per cent noradrenaline
			Noradrenaline	Adrenaline	
1	1	r	2.04	8.36	20
2	300 kg. ♂	l	1.64	8.63	16
3	2	r	1.47	10.47	12
4	450 kg. ♂	l	2.63	7.35	26
Mean			**1.95**	**8.70**	**18.5**

Discussion

It was described by Euler and Hamberg that in using their iodine method the colour tint of the organ extract after oxidation did not always agree with that of the standard. The disagreement of the colour tint may be due to the facts that in the iodine reaction the colour tints of noradrenaline and adrenaline are not always similar and the colour tint due to adrenaline itself varies according to pH of the solution.

In the case of the permanganate method, however, the disagreement of colour tint between the medullary extract and the standard adrenaline solution was unable to find. This coincidence of the colour tint seems to be due to the following facts: that the colour tint of noradrenaline obtained at pH 5.6 is generally similar with that of adrenaline obtained at pH 5.6, and that the colour tints due to adrenaline oxidized at pH 3.6 and at pH 5.6 are almost identical. Noradrenaline can be oxidized at pH 3.6 only less than 10 per cent. Hence, the colour tint of the medullary extract oxidized at pH 3.6 is not influenced so much by the presence of noradrenaline unless the active principle of the extract is mostly noradrenaline, though the colour tint of noradrenaline obtained at pH 3.6 is somewhat different from that of adrenaline.

As regards the noradrenaline content in the adrenals of cattle, some investigations have been reported. Bergström, Euler & Hamberg[1] reported that in the cattle adrenals noradrenaline occurred along with ad-

renaline in a proportion of about 1 : 4. According to Euler & Hamberg,[7] the adrenals of cattle were estimated to contain 0.48–0.70 mg. noradrenaline-HCl and 1.80–2.16 mg. adrenaline-HCl per g. of fresh tissue of the whole gland, and 2.68 mg. noradrenaline-HCl and 11.3 mg. adrenaline-HCl per g. of fresh medullary tissue. In their experiments the relative amount of noradrenaline was estimated to be 23.6% (18.0–27.4%) of the total. Holtz & Schümann[8] found that noradrenaline was contained in the ox and pig adrenals in an amount of about 30 per cent of the total active substances. The relative amount of noradrenaline in the cattle adrenals was estimated by Hermann, Vial & Chatonnet[9] to be 12.6–31.9 per cent. Recently it was reported by Shepherd & West[10] that noradrenaline was contained in the ox and cow adrenals in an amount of 26 and 29 per cent of the total, respectively.

The estimate of the relative amount of noradrenaline obtained in my experiments was, therefore, about the same with those obtained by Holtz & Schümann and Shepherd & West, and somewhat higher than those of Euler & Hamberg and Hermann *et al.*

Summary

1) A method for the differential estimation of noradrenaline and adrenaline by employing the permanganate reagent was described in detail.

2) This method depends on the fact that the two substances are oxidized at pH 3.6 with permanganate in the different oxidation rates. At pH 3.6, adrenaline is completely oxidized within 2 minutes, whereas only less than 10 per cent of noradrenaline can be oxidized during the same oxidation time. At pH 5.6, however, both noradrenaline and adrenaline are fully oxidized within 3 minutes.

3) For differential assay of noradrenaline and adrenaline in a mixture, a sample for assay is oxidized at pH 3.6 for 2 minutes and at pH 5.6 for 3 minutes. By determining the colour densities, the amount of noradrenaline and adrenaline can be calculated from the given formulas.

4) The method has been applied successfully to the medulli-adrenal extracts of cattle and horses. In practising, the colour tint of the extracts and the standard adrenaline solution after oxidation was almost similar.

The noradrenaline content estimated per g. of adrenal medullary tissue was 2.89 mg. (1.02–4.57 mg.) in cattle and 1.95 mg. (1.47–2.63 mg.) in horses. The adrenaline content was estimated to be 7.03 mg. (4.57–9.47 mg.) in the former and 8.70 mg. (7.35–10.47 mg.) in the latter. Therefore, noradrenaline was found in the medullary tissue in an amount of 29% (14–39%) of the total in cattle and of 18.5% (12–26%) in horse.

I wish to thank Prof. T. Suzuki, at whose suggestion this work was carried out, for much valuable advice and encouragement. I am greatly indebted to Drs. T. Kawachi and Y. Baba in Mitsui Institute of Industrial Medicine for practising the spectrophotometric determinations.

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Contribution to the Ocular Manifestation of Riboflavin Deficiency

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The investigation of the pathological changes, particularly, in the visual organ, due to ariboflavinosis (vitamin B_2-deficiency) has been rare and incomplete. Though it is unusual that the deficiency of vitamin B_2 in simple form occurs, the ariboflavinosis seems to happen, not exceptionally, in complication with other various vitamins and nutrient agents, namely in the form of partial phenomenon of polyavitaminosis or malnutrition. For instance the so-called " Shibi-Gattchaki " disease, which is widespread at present in the Tsugaru-area of Aomori Prefecture, Japan, is a kind of nutritive trouble, in which ariboflavinosis and pellagra predominates, as Masuda *et al*[1] have described. It is a subclinical syndrome, consisting of angular stomatitis, glossitis and proctitis. There is also roughness, fissure, edema, erosion of skin and mucous membrane, especially on a boundary part such as the outer pubic region. If the " Shibi-Gattchaki " disease becomes serious then it provokes a psychoneurogenic sign.

Clinical Observation

Thereupon we have carried on research since 1949 on the ocular manifestation in regard to a large number of patients who had complained of foggy vision, twilight blindness i.e. visual disturbance in low illumination. We found some of the following symptoms (by Irinoda[2]) ;

i) angular blepharoconjunctivitis
ii) hyperemia, vascularization, pigmentation at the corneal limbus
iii) superficial diffuse keratitis
iv) nodular formation at the iris
v) temporal pallor, redness, opacity of the optic nerve
vi) retinal changes, such as silver-grayish patches, brownish pigmentation etc.

In order to ascertain the relationship between these ocular signs and riboflavin-deficiency, we have determined the quantity of riboflavin in blood by the fluorometric method, and further measured their saturation into body by a load test. Thereafter we observed the influence upon their recovery by the supply of riboflavin in the body (by Irinoda *et al*[3]). In the first place we recognized that in school-children who seemed to have relatively little nutritive deficiency, the quantity of riboflavin in the blood and urine showed, as a rule, a low rate. In one group of out-

and hospitalized patients, who manifested not only general symptoms but ocular signs and seemed to be somewhat serious, we found a lower rate. Secondly, according to the loading examination, the saturation of riboflavin in body was markedly scanty, when a case of a neuroretinitis with macular lesion was contrasted with a healthy adult, the curve of the total volume of riboflavin-excretion went down considerably, i.e. volume of excretion into urine was very scarce and the excretion-index very small. After the load-test we measured the quantity of riboflavin in the blood of 5 typical cases before, during and after treatment and compared it with the progress of the ocular signs. The results are significant because the low quantity of riboflavin in the blood before treatment, rapidly increased with a large dose of riboflavin, and the ocular signs therewith improved markedly.

From these examinations we deduced that the ocular manifestations of the so-called " Shibi-Gattchaki " disease were for the most part due to ariboflavinosis, and that they contained the symptoms that had close connection with metabolic disturbance of nicotinic acid, thiamine (B_1), adermin (B_6) and other various vitamins. We have investigated the pathohistological findings of these changes.

Pathohistological Findings

Case 1. T. S. 26 year old male. Diagnosis: pannus corneae ariboflavinosus o. utr. (dermatological diagnosis; acne rosacea of 2. grade.)

At both cornea all over the surface, partly interstitially, opaque accompanied with vascularization. Vitamin B_2 quantity in blood 5.2 γ%, N'-methyl nicotinamide in urine 0.30mg%. Histological changes: epithelial cells of the conjunctiva and cornea were multiplied and thickened, besides between epithelial cells considerable vacuole-formation showed. At tunica propria repletion and new-formation of blood-vessels were marked, and at corneal limbus infiltration of histiogenic cells (histiocyte, mónocyte, plasmacyte etc.) extended (Fig. 1, Fig. 2).

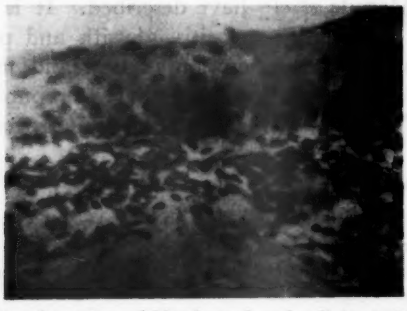

Fig. 1. New-formation of blood-vessel and cellular infiltration at anterior portion of the cornea of man (Case 1).

Case 2. T. N. 2 year old male. Diagnosis; pannus corneae ariboflavinosus o. utr.

At the limbus corneae violent circumcorneal injection showed. Vitamin B_2 quantity in blood 3.0 γ%, N'-methyl nicotinamide in urine 0.21 mg%. Histological changes; epithelial cells of the conjuctiva were markedly multiplied and thickened, besides at their superficial, upper parts keratinization happened, between epithelial cells numerous vacuoles-formation showed. At tunica propria blood-vessels developed, and in its surroundings infiltration of histiogenic cells (histiocyte, monocyte, plasmacyte, fibroblasts etc.) extended (Fig. 3).

Case 3. K. T. 17 year old female. Diagnosis; conjunctivitis catarrhalis acuta cum pig-

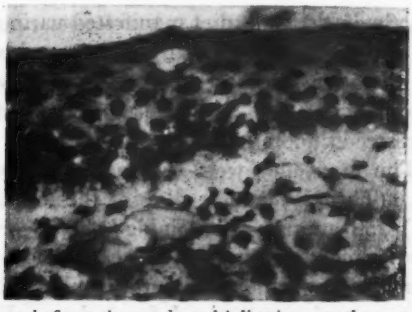

Fig. 2. Vacuole-formation and multiplication at the conjunctival epithelium and histiogenic cellular infiltration (histiocyte, monocyte, plasmacyte etc.) at tunica propria of the corneal limbus of man (Case 1).

Fig. 3

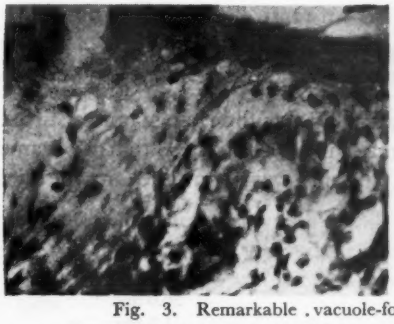

Fig. 4

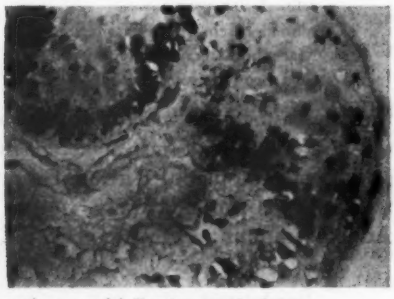

Fig. 3. Remarkable . vacuole-formation, multiplication and hyperkeratinization at the epithelial layer of conjunctiva of man (Case 2).

Fig. 4. Vacuolation of the protoplasms, increase of the keratin and increased melanin-pigment at basal layer of the conjunctival epithelium of man (Case 3).

mentatio limbi corneae o. utr.

Mostly at the lidslitzone and lower part of the corneal limbus, dense deposition of brownish pigment-patch showed. Vitamin B_2 quantity in blood 4.5 γ%, N'-methyl nicotinamide in urine 0.18 mg%. Histological changes; under the basal cell layer of the conjunctiva obvious increase of melanin-pigment and destruction of the epithelial cell, namely bad staining of the nucleus, vacuolation of the protoplasms, and increase of the keratin. That is to say vacuole-formation and keratinization of the epithelial cell layer in two cases, however in the latter, increase of the melanin-pigment, such as at the skin of beginning pellagra (Fig. 4). In sum we saw that the pathohistological view showed the vacuole-formation of protoplasma, the multiplication and keratinization at epithelial cells of the conjunctiva and cornea, the repletion and new-formation of blood-vessel, chronic inflammatory histiogenic infiltration at tunica propria, and sometimes increase of the melanin-pigment at the basal cell layer.

Experimental Study

On the other hand, we have succeeded in an animal experiment that

a rabbit fed on riboflavin-deficient diet manifested various ocular changes.

Method: as experimental animals young rabbits (500–2,000 g. weighed, 1–3 months after birth) were selected. They were kept in an air-conditioned room at 20–25°C in individual metal cages (47×40×37 cm. sized) on wire screen floors preventing them access to the refuse tray and excretions. The food cups were designed to allow feeding without opening the cage. (The care and feeding of the animals have been described by Sato[4].) 58 animals (containing paired control 12) were involved in three experiments during a two-year period.

Dietary procedures: new animals were tuberculin-tested with old tuberculin intracutaneously and quarantined for 5 days. Food was withheld for 24 hours and they were then offered a diet of following composition:

Daily basal diet:

refined rice corn	40 g.
refined casein (by Hammarsten)	10 g.
Maccalum's combined mineral (No. 185)	3.5 g.
soya bean or sesame oil	6.0 g.

these were together with ca 100 cc. water and few filterpaper lumps stirred up, and then boiled in sterilizer 20 minutes in 130–150°C, so that it became soft bread-like food. The necessary vitamins were combined in the diet when it was supplied daily.

Added vitamins: in first experiment

Thiamine	1.25 mg.
Ascorbic acid	20.0 mg.
Pyridoxine	1.0 mg.
Niacin	2.0 mg.
Folic acid	1.0 mg.
Cod liver oil	0.5 cc.

in second experiment besides this

Choline chloride	10.0 mg.
Ca panthothenate	5.0 mg.

in third experiment moreover

Biotin	0.3 mg.
Inositol	0.1 mg.

Further in the diet of the control animal, riboflavin 1 mg. was added daily.

Examination: the animals were weighed daily in the morning, observed on general state and examined weekly with slitlamp, oblique illumination, retionscopy, and as occasion demanded, corneal test by fluorescein-instillation. As the riboflavin measurement by fluorometric method

was used, the catheterized urine and autopsied tissue, and load test of riboflavin, the recovery test by riboflavin-supply was carried out, too. Before the animals died, they were butchered and autopsied to prevent post-mortem change. Various organs were measured for riboflavin-quantitification. Tissue fragments (visual organ, liver, brain) were fixed in 10% formalin, and then embedded in paraffin, celloidin, fleezing and stained by H. E., V. G., Sudan III, mucicarmin, thionin, Kultschitzky's myelin-staining technique, Suzuki's axon cylinder-staining technique, Marchi's technique (by Osmium acid-fixation).

Results: From those experiments we obtained the following results:—

1) All rabbits suffered from such general symptom as loss of hair, brown stains of fur, slow rate of growth, diarrhea and anorexia, after 15–40 days (23 days on an average).

2) The rabbits deficient in riboflavin made a much smaller urinal excretion of riboflavin than the control group, and they showed a remarkably lower rate of urinal excretion in the riboflavin load test, indicating a smaller degree of riboflavin-saturation in the body.

3) In the visceras (liver, kidney, lung, heart) the quantity of riboflavin was smaller than in those of the control animals whether the former were loaded with riboflavin or not.

4) It was observed, both clinically and pathohistologically, that the symptoms of deficiency were well cured by injection of riboflavin.

5) In cases of only deficiency in riboflavin, 5 of 26 cases showed diffuse superficial opacity of the cornea, and 3 showed coneal vascularization.

6) Out of 20 cases of deficiency in several vitamins of VB_2-complex, 3 showed diffuse superficial corneal opacity, 1 erosio corneae, 4 corneal vascularization and 1 edematous opacity of the retina.

7) Pathohistological findings from these clinical signs were as follows;

a) The edema and the vacuoles of the corneal epithelial layer, vascularization in the stroma below the epithelium, and subepithelial cellular infiltrations (histiocyte, monocyte, plasmacyte, etc.) that indicated chronic inflammation at the corneal limbus were found. (Fig. 5, Fig. 6, Fig. 7) b) In one case of the erosio corneae we found necrosis and falling of the corneal epitheliums and polynuclear leucocyte-infiltration between the upper lamellæ of the corneal stroma. c) In the case of the retinal edematous opacity we found the degeneration-substance like mucin (postive by the mucicarmin-staining) at the retinal nerve-fiber-layer. d) In most of the cases signs indicating the process of degeneration of the retinal ganglion cells, namely chromatolysis and chromatophilia of the Nissle's body, and alveolar vacuole-formation of the protoplasma were

Fig. 5. New-formation of blood-vessel at anterior portion of the cornea of rabbit.

Fig. 6. Remarkable vacuole-formation at epithelial layer of the cornea of rabbit.

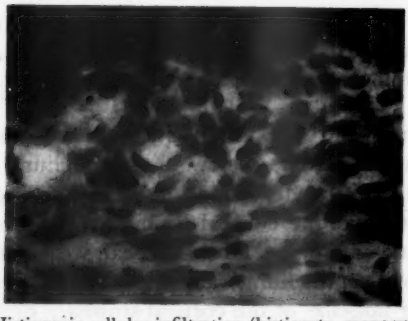

Fig. 7. Histiogenic cellular infiltration (histiocyte, monocyte, plasmacyte etc.) at tunica propria of the corneal limbus of rabbit.

found. Such changes were found at the ganglion cell in the cortex of brain, too. (Fig. 9, Fig. 10, Fig. 11, Fig. 12, Fig. 13) e) In the liver central fatty degeneration was marked. (Fig. 14).

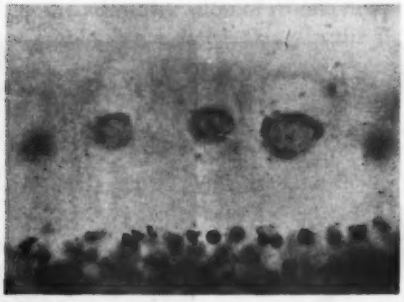

Fig. 8. Nissle's body at the retinal ganglion cell of contrast-animal (by thionin-staining).

Fig. 9. Chromatophilia at the retinal ganglion cell of ariboflavinotic animal.

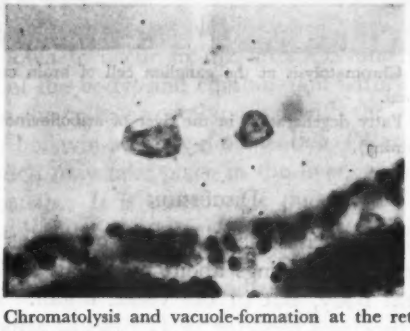

Fig. 10. Chromatolysis and vacuole-formation at the retinal ganglion cell of ariboflavinotic animal.

Thus we recognized that with a lack of riboflavin there occurs pathological changes, chiefly at the cornea and the retina, and then a decrease of visual acuity. We believe, therefore, that we have sufficient data to explain the clinical symptoms of riboflavin-deficiency in man.

Fig. 11

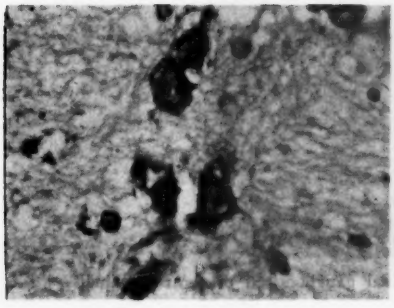

Fig. 12

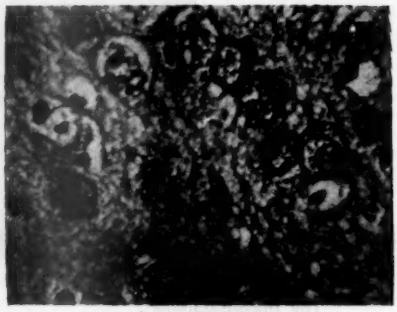

Fig. 11. Nissle's body at the ganglion cell of brain cortex of contrast-animal.

Fig. 12. Chromatophilia and alveolar vacuole-formation at the ganglion cell of brain cortex of ariboflavinotic animal.

Fig. 13

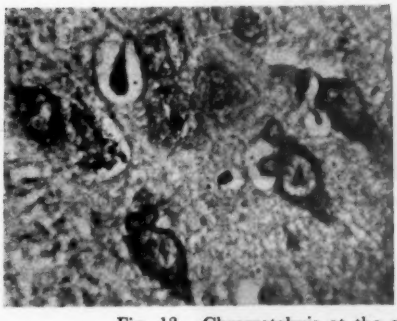

Fig. 14

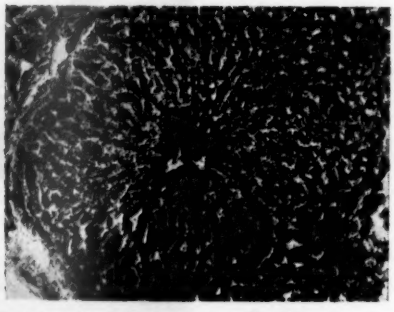

Fig. 13. Chromatolysis at the ganglion cell of brain cortex of ariboflavinotic animal.

Fig. 14. Fatty degeneration in the liver of ariboflavinotic animal (by Sudan III staining).

Discussion

Our observation indicates that in human beings, as well as in animals, the dificiency of riboflavin brings about numerous and various signs, not only in the general body, but in the visual organ. Previously, Sydenstricker[5] and his associates reported in 47 cases of ariboflavinotic patients with cheilitis, glossitis, seborrhoea ocular manifestation such as 37 conjunctivitis, 45 circumcorneal injection, 37 corneal vascularization, 18 corneal opacity, 19 pigmentation of the iris, 4 iritis, 6 cataract, and H. J. Stern, Landau,[6),7] J. J. Stern[8] observed that in ariboflavinosis, vascularization of the cornea (so-called eczematous keratitis) often occurred, which was

reactively influenced by the administration of riboflavain. Experimentally, Day and O'Brien[9] by employing a diet lacking in the B_2 group, especially riboflavin, produced blepharitis, conjunctivitis, interstitial vascular keratitis and cataract in the rat. Although others have been unable to confirm, this observation led to corneal vascularizations in man being regarded as a riboflavin deficiency. Later, Bessey and Wolbach[10] brought out corneal vascularity and Suda[11] brought out diffuse superficial keratitis in white rats bred with riboflavin-deficient diets.

Clinically, in riboflavin deficiency the following ocular symptoms were recently described; smarting, itching feeling as of the presence of a foreign body, and moderate photophobia; objectively, there are found injection of the bulbar conjunctiva, vascular keratitis, congestion of the iris and visual disturbances. The keratitis begins with engorgements of the perilimbal group of vessels, which is followed by proliferation of the capillaries in the superficial and middle corneal strata and interstitial infiltration. The vascular condition is attributed to the need of the cornea to obtain from the vessels the oxygen which it lacks, owing to the deficiency of the yellow respiratory ferment of Warburg (of which flavine is a component).

These views, however, are not yet fully confirmed. Some authors have observed an improvement in the corneal lesions through riboflavin administration. Others affirm that corneal vascularization is not a sure sign of deficiency of vitamin B and deny altogether any relation between perilimbal engorgement of vessels and vitamin deficiency. If this is so, what are the reasons for the occurrence of these signs in the visual organ? When one follows a molecule of riboflavin from its entrance into the body to its final position in cellular oxidation, one finds that it has to undergo numerous changes before it is capable of entering reactive processes, i.e. phosphorylation, known to occur in the intestines and possibly also performed in the cells of the body, and combination with a protein, believed to take place within the cells, or combination with the remaining portion of the Hogness's riboflavin-adenine-dinucleotide (Warburg's yellow enzyme) complex, which may take place in the liver, with subsequent combination with a protein. It is conceivable that a defect in the chain of oxidative reactions other than in the metabolism of the riboflavin-containing enzyme may be present. This defect may conceivably be in the reducing system (coenzyme I and II) or in the oxidizing system (cytochrome C, succinate-fumarate system, hexose monophosphate, l-, d-aminoacid, hypoxanthine, xanthine, certain aldehydes). That is to say, the recent investigations on oxidative enzymes which contain riboflavin indicate that there exists, in addition to the yellow enzyme, which acts as a hydrogen acceptor in oxidation of carbohydrate, yet other riboflavin-protein complexes (amino-acids) which act as activators of the reduced coenzyme. So

the end results of riboflavin deficiency would not be affected by these newer concepts, for such a deficiency would result in a severe disturbance of the oxidative systems in the body.

From the preceding consideration, it can be seen that the function of the yellow enzyme is to aid in the transfer of hydrogen from the tissue cells to the blood stream, where free oxygen is available from the breakdown of oxyhaemoglobin to hemoglobin, and where the oxidation of the hydrogen received originally from the substrate (hemin anolog chlorophyll), through the coenzyme, can be accomplished. It is believed that if hemin substances are not present in a tissue (such as the avascular cornea) the oxidation within the cell is accomplished by Warburg's yellow enzyme. Because in avascular or hypovascular tissue such as the cornea, inner layer of the retina hemin substances are not brought into close proximity with the tissue cells, so it may be assumed that the greater part of gaseous interchange in oxidation is effected through Warburg's yellow enzyme. Therefore, in the avascular or hypovascular tissue riboflavin is deficient, so local asphyxia is brought about and at first degenerative changes occur. Further, on the other side, as an attempt to overcome local asphysia by bringing available oxygen into closer proximity with tissue cells, the proliferation of capillaries was brought out.

We are going to suggest that in the event of a deficiency in any of these elements, or in some one of the elements, namely, riboflavin, the capillary endotheliums will be one of the first, if not the first, of the tissues to suffer the effects of interference with normal respiration and metabolism. The result of the anoxia upon the capillaries is a derangement of function, which we shall refer to as " capillary dysergia " with metabolic disorder of the cells of the neighbouring tissue. So accompanied with these changes, chronic inflammation was involved as a consequence.

Hitherto nothing has been described about the retinal changes due to ariboflavinosis. In experimental study we saw a case of retinal edematous swelling with pathohistological mucous degeneration of nerve-fiber-layer due to lack of riboflavin and some of the other factors of the vitamin B_2 group. Excepting the one above, all the cases without the ophthalmoscopical finding in the ganglion cells, showed remarkable degeneration such as chromatophilia or chromatolysis of the Nissle's body and alveolar vacuole-formation of protoplasms. These changes of the retinal ganglion cell must be discerned as the beginning stage of degeneration, caused by the nutritional deficiency of the cells in riboflavin, because the same changes occurred in the ganglion cell of the brain cortex by the same cause.

Those changes should cause various visual disturbances such as " nutritional amblyopy " (Moore,[12] Landor and Pollister[13], Scott[14], Rodger[15] and " Twilight-blindness " (already described by Pock-Steen[16]

and also clinically noticed by us). Besides if reconsidering the hyperkeratinization of the corneal and conjunctival epithelia, which were shown in our clinical cases, we found great similarity in nature between the ocular lesions of avitaminosis A and ariboflavinosis. The former, however, responded to vitamin A. As the processes in conjunctivitis or avitaminosis A extend through the corneal epithelium and down into the cornea, it is not surprising that they disturb the cornea with a typical, but probably secondary, response like that seen in ariboflavinosis. Since the site of the first reaction of riboflavin is just beneath the corneal epithelium, it is possible to conceive how riboflavin may thus be secondarily disturbed. As the process reaches the lamellae just under the corneal epithelium, it is possible that the keratitic manifestation represents a secondary riboflavin distrubance. In consequence, avitaminosis A concurrent with ariboflavinosis might be expected to add to the extent or intensity of the keratitis due to the ariboflavinosis. And according to Spies *et al*[17] the enzymatic function of riboflavin has so a close connection with other various vitamins, that nicotinic acid in the form of its amide is an important factor in forming the co-zymase (von Euler) and co-ferment (Warburg). Thiamine is also an important co-enzyme in phosphorized thiamine (cocarboxylase). So ariboflavinosis may happen as a partial phenomenon of polyavitaminosis.

Hills *et al*[18] have reported on the experimental deficiency of riboflavin in the human being. Under the conditions of their study the tendency of small vascular twigs in the margins of the cornea to disappear and reappear months later was frequently noted, but no proliferation of the vessels of the limbic plexus was seen. Certainly these must have been a latent ariboflavinosis, if they become manifest by the joining of other factors, such as injury and light exposure. The " yellow enzyme " is necessary in such a large number of cellular reactions that it is surprising that abnormalities beneath the dermal layer were absent or minimal. The inability to find any changes in the metabolites in the body fluids may be because the cell, once matured, requires only a minimum amount of riboflavin. However, the rate of replacement of such cells with others may be dependent on a more abundant supply of riboflavin, obtainable either from the diet or through the breakdown of less vital tissue.

Summary

By clinical observation on so-called " Shibi-Gattchaki " patients and the experimental study on young riboflavin-deficient rabbits we have obtained the following results;

1. The deficiency of riboflavin mostly caused degenerative changes (vacuolar degenerations), regenerative process (compensatory vascularization) and reactive chronic inflammation in the avascular or hypovascular

organ such as the cornea, inner layer of the retina.

2. Ocular manifestation of ariboflavinosis including its latent state occurred considerably frequently in human beings as well as in animals, as compared with other signs.

3. Ocular symptom, like other various clinical signs, are pathognomonically reliable.

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